CHANGES IN ABSCISIC ACID CONCENTRATION DURING ZYGOTIC EMBRYOGENESIS IN LOBLOLLY PINE (*PINUS TAEDA*) AS DETERMINED BY INDIRECT ELISA

A Dissertation Submitted by

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LIST OF EQUATIONS

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LIST OF ABBREVIATIONS

ABA	abscisic acid
ABA-BSA	abscisic acid-bovine serum albumin conjugate
ABA-C_4-ABH-BSA	same as ABA-BSA
ABH	aminobenzoyl hydrazide
AcOEt	ethyl acetate
BB	borate buffer
B_i	% binding of standard/sample
B_o	0% binding of antibody
B_{max}	100% binding of antibody
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
CI	confidence interval
CPM	counts per minute
CR	concentration ratio
DSA	days since anthesis
DDC	diethyldithiocarbamate
DMSO	dimethyl sulfoxide
DPM	disintegrations per minute
DSF	days since fertilization
ELISA	enzyme-linked immunosorbant assay
GA	gibberellins

GAM-biotin	goat anti-[mouse antibody]-biotin conjugate
GC/MS	gas chromatography/mass spectroscopy
	-SIM selected (or single) ion monitoring
	-SCAN all ions
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
H_2SO_4	sulfuric acid
K	equilibrium constant
LEA	late embryogenesis abundant
mAb	monoclonal antibody
Me-ABA	methyl-ABA
MeOH	methanol
MeOOH	methyl formate
MSD	mass-selective quadrapole detector
MW	molecular weight
$NaNO_2$	sodium nitrite
O.D.	optical density
PAR	peak area ratio
PEG	polyethylene glycol
PGH	plant growth hormone

PGR	plant growth regulator
PGS	plant growth substance
PNPP	*p*-nitro-phenyl phosphate
ppm	part per million
RO	reverse osmosis water
RT	room temperature
R_t	retention time
SOD	stage of development
Strep-HRP	streptavidin-poly-20HRP
TMB	tetramethyl benzidine
UC	Union Camp
	UC_{91} - 1991 growing season (genotype 10-68) UC_{92} - 1992 growing season (genotype 10-84)
UV_{254}	ultraviolet at 254 nm
WA	Weyerhaeuser mother tree A
	WA_{91} - 1991 growing season WA_{92} - 1992 growing season WA_{93} - 1993 growing season
WB	Weyerhaeuser mother tree B
	WB_{91} - 1991 growing season WB_{92} - 1992 growing season

LIST OF ABBREVIATIONS (cont.)

WV	Westvaco
	WV_{91} - 1991 growing season (genotype 240) WV_{92} - 1992 growing season (genotype 8)
α	confidence level
θ	equivalence region

It is evening, and children slowly dream away the storms of day.
It is evening, and stars glow gently in the quiet heavens.

Can we understand a dream?

Find a net to capture the meaning of a glowing star?

What bridge spans the vast space we must cross to reach understanding?

How small are we who attempt the journey! And yet somehow we learn to find our glory in a brave and endless struggle to comprehend eternal mysteries. We are voyagers in an infinite sea, our destination always beyond the horizon. But we are voyagers.

-- CCAR

ABSTRACT

No studies published to date have documented changes in endogenous ABA levels in zygotic conifer tissues. This research examined fluctuations of endogenous (+)-abscisic acid (ABA) in embryos, megagametophytes, and suspensors during zygotic embryogenesis in loblolly pine (*Pinus taeda*). Methods were developed to collect and store seed tissues, to extract and purify ABA, and to quantitate ABA with an amplified indirect enzyme-linked immunosorbant assay (ELISA). The indirect ELISA, developed with a commercially available monoclonal antibody and amplified using an avidin-biotin-multiple horseradish peroxidase, provided reliable and sensitive (~2 pg/100 μL) estimates for ABA in loblolly pine zygotic tissues. Validation of early, mid, and late zygotic tissues using definitive gas chromatograph/mass spectroscopy (GC/MS) ruled out the presence of interfering substances.

Commonly used methods used were found to influence the final ABA estimates. Separation of embryo, suspensor, and megagametophyte tissues during dissection significantly increased (~50%) the endogenous ABA levels. ABA levels were also increased (~28%) during tissue storage prior to analysis despite stringent storage conditions. Therefore, absolute ABA estimates must be considered carefully, although the overall trends should be accurate.

Results indicated two significant peaks in endogenous ABA levels (dry-weight basis) in the whole ovule. The first major peak occurred early in development and was attributable to megagametophytic ABA. The second peak occurred at mid-development and was attributable to embryonic ABA. Similar to previously documented trends in cereals, legumes, and woody angiosperms, embryonic ABA (dry-weight and per embryo basis) was

extremely low early in development, significantly higher during mid-development, and then rapidly declined to low levels at seed maturity. This trend was found to be similar in four mother trees during the 1992 growing season and across two growing seasons (1992 and 1993) in a single mother tree. Such findings suggest that environmental factors (such as day length) that are common within the geographic regions are critical in regulating endogenous ABA levels. Moreover, it appears that within-species genetic variations play a minor role.

Several causal relationships can be inferred from endogenous ABA levels and physiological changes in loblolly pine zygotic tissues. First, embryonic ABA increased prior to and concurrent with the accumulation of dry-weight in the embryo. This supports the hypothesis that high levels of embryonic ABA may initiate the production of storage products, but are not necessarily required for their continued synthesis. Second, megagametophytic ABA levels and the percent moisture of the megagametophyte and embryo all dropped rapidly after fertilization. This suggests that megagametophytic ABA may initiate and control the rate of desiccation in both tissues. Third, embryonic ABA (μM basis) increased rapidly during late development and increased to an extremely high level at complete seed dormancy. This suggests that a high level of embryonic ABA may be correlated with the suppression of precocious germination as well as induction and maintenance of dormancy in embryos of loblolly pine.

CHAPTER ONE - LITERATURE REVIEW

PINUS ZYGOTIC EMBRYOGENESIS & PLANT GROWTH HORMONES

Zygotic embryogenesis can be divided into two stages: 1) a short proembryo phase occurring in the archegonium, and 2) an embryo phase occurring after the proembryo elongates into the megagametophyte. The proembryo phase occurs immediately after fertilization and involves the zygote dividing to form the tier cells, i.e., open, rosette, suspensor, and apical. The suspensor cells elongate pushing the apical tier cells through the archegonium wall and into the megagametophyte tissue. The apical tier cells subsequently divide to form embryonic tube cells, which elongate further into the megagametophyte, and distal apical cells (proembryo). Breakdown of the megagametophyte supplies nutrients to the developing embryo with consequent formation of the corrosion cavity. Formation of the corrosion cavity, thus, marks the end of the proembryo phase (1,2).

Pinus can undergo two types of polyembryony: simple and cleavage. Simple polyembryony occurs when more than one egg per ovule is fertilized. In this case, one pro-embryo eventually dominates and continues to develop. In cleavage polyembryony, the distal apical cells divide into four sets of cells each developing into separate, but genetically identical, embryos. Again, one embryo eventually dominates and continues to develop while the other embryos degenerate.

Continued division of the dominant apical cell leads to formation of a small club shaped embryo-proper as the suspensor cells push it further into the corrosion cavity of the

megagametophyte (1,2). At this point, the embryo rapidly increases in size through accumulation of storage products in the form of lipids, starches, and proteins (3). Cells at the distal end eventually form the cotyledons around the dome-shaped apical meristem, while the root apex forms below the apical meristem. The embryonic axis elongates between the shoot and root apical meristems and the root cap forms before the embryo reaches maturation and subsequently becomes dormant (1,2).

It is clear that all aspects of this highly complex development process proceed in a finely regulated manner, and to a large extent, this precise regulation is very likely achieved through the actions of minute quantities of substances called *plant growth hormones* (PGHs). PGHs are extremely important agents in the overall coordination and integration of developmental processes such as cell division, expansion, and elongation, in different regions of the plant. They are also involved in the response of plants to their physical environment, e.g., temperature, light intensity, day length, soil moisture, humidity, and nutritional factors, as well as the regulation of genetic expression of such processes as germination, transpiration, and protein synthesis. PGHs can, in a sense, act as chemical messengers passing from one cell, tissue, or organ, to another, thereby providing a method of communication between different parts of the whole plant (4,5,6).

The term "hormone" originates from the Greek language where it means "arousing to activity", and it was first used in animal physiology to identify chemical messengers (4). Its original use in plant physiology was derived from this mammalian concept, but the principle differs greatly since tissue development in plants is far less complex than in animals

(7). This has lead to heated debates between physiologists as to whether the term PGH is accurate or if *plant growth regulators* (PGR) or *plant growth substances* (PGS) should be used to identify these agents since they do not follow all the properties of an animal hormone, e.g., transport from a synthesis site to an action site. It is the contention of Davies (8) that the term PGH should be used with all its imperfections, "We must break with the characteristics expected of animal hormones: plant hormones are a unique set of compounds, with unique metabolisms and properties ... In fact, (the) notion of a plant hormone is much closer to the meaning of the Greek origin of the word *hormone*... (as this term does not imply) that transport or action at a distance is ... (essential)." Therefore, PGH will be retained in this dissertation.

The definition of PGHs is an operational one, i.e., they are defined in terms of their biological activity rather than their chemical nature (5). PGHs are broadly defined as having the following attributes:

1) known chemical structure,

2) are organic substances other than a nutrient or vitamins which by definition supplies carbon and energy or essential mineral elements, respectively,

3) are biosynthesized in some plant organ(s),

4) are usually translocated within the plant from a biosynthesis site to an action site,

5) have specific biological activity in extremely low concentrations (<< 1 mM),

6) performs a fundamental role in regulating biochemical, physiological, or morphological activity *in-vivo*,

7) broadly distributed throughout the plant kingdom (7),

8) active in tissues where they are synthesized, as well as in distant tissues, and

9) modulate the action of other hormones (5,9).

The established PGHs, auxins (specifically, indole-3-acetic acid or IAA), cytokinins, gibberellins (GAs), ethylene, and abscisic acid (ABA), are associated with a wide variety of biochemical, physiological, and morphological functions. Currently, there are three other groups of substances that are considered hormones because of their wide-spread distribution in the plant kingdom and their specialized biological activity: polyamines, brassaninolides, and jasmonates. All of these PGHs have been identified in seeds, and the established PGHs show significant changes in concentration during the course of zygotic development in various seed tissues and fruit, suggesting a critical role in the final outcome of embryo development.

The original goal of this thesis was to assist in obtaining somatic embryogenesis in loblolly pine, a process that has so far proven very difficult to develop to date. It was thought that a better understanding of the natural levels of PGHs during zygotic embryogenesis (zygotic referring to the embryo, suspensor, and megagametophyte) would prove valuable in the rational preparation of media used to obtain normal maturation and development of loblolly pine somatic embryos. This idea was supported by Attree and Fowke (10) who stated, "In order to encourage the production of mature developmental stages of conifer somatic embryos, ... growth conditions have been used (which) probably approximate the natural *in ovulo* conditions existing during early embryo development."

At the start of this research, experimentation at the Institute of Paper Science and Technology (IPST) was centered on developing a better understanding of the processes of embryo maturation and development in loblolly pine. A survey of the literature (forthcoming) indicated that ABA was, in almost all cases, required for continued normal development of somatic embryos in both angiosperms and gymnosperms. Therefore, ABA was chosen as the first PGH to study during zygotic embryogenesis in loblolly pine. The next consideration was the most appropriate technique for quantitation of ABA in loblolly pine seed tissues. A review of these current ABA quantitation methods is presented in the following section.

ANALYTICAL METHODS FOR THE QUANTITATION OF ABSCISIC ACID

Techniques for extraction, purification, and quantitation of ABA have been reviewed several times in the past eight years (11,12,13,14,15,16). Immunoassays and chromatographic techniques were compared by Chan (17).

Tissue Sampling Techniques

For accurate quantitation of ABA, tissue sampling techniques must: 1) prevent changes in the post-harvest ABA concentration, 2) avoid the formation of diffusion products, and 3) allow a measure of the amount of tissue sampled (18). It is well known that endogenous ABA levels in water-stressed tissues can increase rapidly (80-fold or more) in a very short period of time (19) either by direct synthesis of ABA or by hydrolysis of ABA-conjugates. Ideally, tissues should be frozen as soon as they are harvested, preferably in liquid nitrogen, and stored at -20°C (13) or lower. Sometimes the frozen tissues are lyophi-

lized prior to storage at -20°C or lower (16). Heat and bright light should also be avoided (16) as ABA easily undergoes enzymatic and chemical oxidation and isomerization when exposed to these conditions. Thus, although many researchers have reported performing tissue dissections at room temperature (RT) under normal lighting, cold room conditions have been preferred (20,21).

Extraction Techniques

Various solvents have been used to extract ABA from plant tissues. As with the sampling techniques, it is critical that extraction methods do not alter the existing endogenous ABA level by degrading ABA or rearranging ABA-conjugates. The most frequently used solvents include neutral or acidified (pH adjusted) absolute or 80-90% (v/v) methanol or acetone solutions as plant enzymes are inactivated by these solvents (16). Water and aqueous buffers have been used to a lesser extent, but these may promote enzymatic breakdown of ABA (22).

Absolute methanol was reported superior to absolute acetone in extracting IAA from *Zea mays* (maize) seeds and pine shoots. The addition of 10% water to absolute methanol or acetone improved the extracting power of both solvents (22). One concern with methanol is the potential for release of ABA from conjugates by alkaline hydrolysis or trans-esterification of conjugated ABA to methyl-ABA. Under acidic conditions, methanolysis of ABA-glucose ester to methyl-ABA was slower, but the potential for methylation of the ABA carboxylic group increased (13,16,23). For these reasons, neutral pH-adjusted methanolic solutions (80-90% methanol) are used most often.

Although it has not been proven that antioxidants prevent the breakdown of ABA, they have been shown to be highly effective in reducing the breakdown of IAA; increases of 8-37% recovery have been reported when using an antioxidant during IAA extractions (22,24). For this reason, antioxidants have been used during the extraction of ABA. The most commonly used antioxidant is BHT (butylated hydroxytoluene), but in a few cases, BHA (butylated hydroxyanisole) and DDC (sodium diethyldithiocarbamate) have been used (11,13). A survey of the literature showed that there was no recommended concentration for these antioxidants; concentrations ranged from 20 to 2000 mg/L with 100 mg/L being most common.

The period for extraction varied greatly, from 1-2 minutes with violent grinding of the tissue in extracting solvent (11,13) to as long as 5 days with slow stirring (25,26) followed by filtration. The majority of researchers reported extracting tissues overnight or 24 hours with continual mixing. Lastly, as with tissue sampling, extractions must be performed at low temperatures (4°C) and under minimal lighting or darkness to prevent ABA decomposition and the isomerization of the natural 2-*cis* double bond of ABA to its unnatural 2-*trans* form (13,16).

The most pertinent question in hormone extraction, and the most difficult to assess, is how efficient is the solvent at extracting ABA from plant tissues? Since the amount of ABA present in the tissue is unknown, it is difficult to determine the absolute recovery of the extraction method. To monitor purification losses, a small amount of radioactive ABA ([^{3}H]- or [^{14}C]-ABA) is added to the extraction solvent as an internal standard. Losses of sample

ABA during purification are unavoidable and can be considerable. These losses can be due to adsorption to glassware, incomplete recovery from chromatographic materials, incomplete methylation, etc. Also, ABA losses between sample extracts can vary considerable and are not necessarily related to the amount of ABA present in the extract. Therefore, recoveries must be determined for every extract. It is especially critical that these losses be taken into account when quantitation is required (13).

Purification Techniques

The type and degree of purification required for the isolation of ABA depends greatly on the choice of plant tissue because the tissue type will determine the type and amount of contaminants present. Successive approximation and validation techniques are essential in determining the level of purification required for each plant tissue and quantitation method. Successive approximation uses a series of different, and increasingly more rigorous, purification steps to determine the degree of purification required. It is assumed that if additional clean-up steps do not lead to further increase in recovery, then the sample is clean and the analysis accurate for the quantitative techniques and tissue utilized. Validation of the routine quantitative technique by a second definitive method, such as GC/MS, is then irrefutable evidence of suitable purification (27,28).

In the past, a multitude of purification methods have been used for analyzing ABA and it would be arduous to cover all the methods; therefore, only the commonly used methods will be discussed in this section. The reader is referred to the references at the beginning of this section for a more extensive review. The most popular methods for purifying ABA are

filtration and/or centrifugation and solvent partitioning as preliminary purification steps, typically followed by chromatography using pre-packed C_{18} columns and/or HPLC as final steps.

Filtration and/or centrifugation after extraction will remove insoluble materials, such as plant cell walls and high molecular weight proteins, lipids, and chlorophylls with minimal loss of ABA. Filtration is accomplished through a multitude of media, including filter paper (29,30), nylon (31), or Miracloth (31). Allowing the extract to stand at low temperature overnight often induces lipids to precipitate (16). Freezing and thawing of aqueous extracts followed by centrifugation is also effective in removing precipitated lipids (16,32,33).

Roto-evaporation *in-vacuo* (29) or evaporation under a stream of nitrogen gas at 30-40°C are techniques used to remove organic solvents and reduce extract volume while minimizing the breakdown of ABA. Most researchers report evaporating sample extracts after filtration and/or centrifugation (31), although in some cases evaporation was used immediately prior to HPLC purification or quantitation (30).

Solvent partitioning between two immiscible solvents is often used as a preliminary purification step, especially when dealing with large extract volumes containing a great deal of contaminants. Partitioning can considerably reduce the dry weight of an extract, but has the disadvantage of generating large volumes of hazardous wastes with the potential for spillage and the need for proper disposal. Also, the use of an internal standard is critical when using solvent partitioning as recovery rates can be low.

ABA is a weak acid having a pK_a of 4.8 which makes its distribution between water and various solvents to be pH-dependent. The most reliable partitioning system is an alkaline extraction (pH 7.5-9.0) against ether or ethyl acetate to remove neutral and basic organic-soluble contaminants. Under these conditions, ABA is almost completely dissociated and virtually insoluble in organic solvents. Acidification of the extract (pH 2.5-3.0), followed by multiple partitioning against ether or ethyl acetate, will remove most acidic water-soluble contaminants (ABA is almost completely undissociated under these conditions and is soluble in organic solvents).

The use of pre-packed C_{18} columns has been very popular in ABA purification either alone or as a "clean-up" prior to reverse-phase HPLC. Similar to solvent partitioning, these columns take advantage of ABA's solubility and polarity in solvents at particular pHs. These columns are simple to use, disposable, possess good separatory efficiency and high sample capacity, reduce sample preparation time, require only small volumes, and can yield nearly 100% recovery of ABA. An excellent discussion as to the versatility of these columns in removing contaminants was given by Parry and Horgan (11). The extract, in a highly polar solvent (e.g., 20% methanol), was eluted through a pre-conditioned C_{18} column. At this concentration of methanol, ABA (much less polar than the solvent) was retained on the column matrix, and the highly polar compounds are removed from the column by washing with additional 20% methanol. ABA was then selectively removed from the column with an appropriate solvent concentration, i.e., 32-55% methanol (slightly polar solvent), while less polar materials remained bound to the column matrix.

High-Performance Liquid Chromatography (HPLC) has become the most widely used method for rigorous purification of ABA. The advantages of HPLC include high resolving power, reproducibility, and reduced analysis time. Multiple compounds can be analyzed during a single run with virtually quantitative recovery. The variety of packing materials and solvent systems are too numerous to discuss here and the reader is again referred to the reviews listed at the beginning of this section (11,13). The most widely used method is reverse-phase C_{18} HPLC employing a gradient system of methanol, ethanol, or acetonitrile in acidified water coupled with UV detection at 254 nm (UV_{254}). This system relies on a non-polar stationary phase (C_{18}) and a mobile phase of changing polarity to manipulate the retention times of the solute components. Again, as with solvent partitioning and the pre-packed C_{18} columns, this method takes advantage of the solubility and polarity of ABA at particular pH values to control separation from its contaminants (11,13).

Quantitation Techniques

The most reliable and extensively used physico-chemical techniques for ABA quantitation is *G*as Chromatography (GC) coupled with one of the following detector types: *F*lame *I*onization (FID) or *E*lectron *C*apture (ECD) for quantitation, or *M*ass *S*pectrometry (MS) for identification and quantitation. These methods of detection demonstrate high selectivity and sensitivity towards ABA and its conjugates. *H*igh-*P*erformance *L*iquid *C*hromatography (HPLC) with ultraviolet (UV_{254}) detection is also commonly used for quantitation, but suffers from lack of sensitivity and specificity as multiple compounds may exhibit identical retention times. Because GC and HPLC are subject to a variety of interfer-

ences, they generally require extensive clean-up procedures prior to quantitation. Since ABA is a minor component in plant tissues, solvent partitioning, column chromatography, and preparatory HPLC have all been used to remove contaminants from plant extracts. These quantitation methods are, therefore, very labor-intensive, and in many cases require derivitization of ABA. Also problematic is the demand for large quantities of tissues to make up for the poor recovery of ABA that unavoidably occurs during extensive purification. Chromatographic systems generally require highly trained personnel and costly equipment, but have advantages in their capability to measure multiple compounds in the same analytical run, ready automation, and reliance upon inexpensive reagents (11,13,17).

With the development of polyclonal and monoclonal antibodies of high specificity and affinity, immunoassays, such as *R*adio-*I*mmuno*a*ssay (RIA) and *E*nzyme-*L*inked *I*mmuno-sorbant *A*ssay (ELISA), provide rapid, specific, and highly sensitive (in comparison to chromatographic) methods for quantitating ABA in plant tissues. As their names imply, RIAs utilize radioisotopes, such as ^{3}H-, ^{14}C-, or ^{125}I-labeled antigens or antibodies, as a means of detection and are quantitated using an scintillation counter. ELISAs are based on spectrophotometric assays of enzymes which have been covalently coupled to antigens or antibodies. Polyclonal antibodies are directed against more than one antigenic determinant, but are produced directly from the sera of immunized animals, e.g., goat, mouse, and rabbit. On the other hand, monoclonal antibodies are directed against only one determinant and are produced using elaborate hybridoma techniques (34). Monoclonal antibodies have the following advantages over polyclonals: 1) unlimited supply with constant properties; 2) recognition of a single antigen site, e.g., ABA molecule; 3) impure antigens or antigen mixtures can be used

for immunizations, e.g., (±)-ABA; 4) few or no cross-reactions due to low-affinity interactions; 5) equally effective in RIA or ELISA; 6) rapid equilibrium in binding the antigen; and 7) infrequent inhibition of the biological activity of the antigen (35).

These techniques typically do not require as much purification as HPLC and GC since most contaminants do not interfere with the antibody-antigen interaction, but this must be validated using physico-chemical methods, e.g., GC/MS. Because of the specificity and sensitivity of the antibody binding, as well as the higher recovery rates (due to minimal clean-up requirements), less tissue is needed for accurate and precise immunological quantitation (11,12, 13,14,15,16). Technical expertise is not as demanding for immunoassays as for chromatographic methods, and instrumentation is less costly and easily automated, typically requiring only pipettors and a simple spectrophotometer or microplate reader. Immunoassays do require relatively expensive reagents in the form of antibodies, antigens, and various protein conjugates, some of which may not be commercially available and thus require time and expertise to synthesize, purify, and test for specificity. Analysis of multiple analytes is not possible in an immunoassay (17), and accuracy of the assay must be validated using an independent technique, such as GC/MS. Immunoassays, being a "biological" method, are prone to inhibition by organic solvents and buffer conditions. However, unless new tissues are assayed or extraction/ purification methods are altered, a single validation of an immunoassay is usually sufficient (11). A further disadvantage resulting from the "biological" nature of the ELISA is that estimates from even a well operating assay may be imprecise due to high variability. Therefore, multiple extractions of plant samples and large sample sizes may be required to overcome the inherent variability of the assay.

Although the operating principles behind RIA and ELISA are very similar, there are many factors favoring the use of ELISA: 1) the enzyme continues to build up a product improving detectibility (RIA labeled species only give one decay signal; 2) ELISA does not require expensive equipment, only a spectrophotometer; 3) ELISA does not require licensing for radioactive materials and therefore does not entail the same disposal problems as RIA; 4) ELISA uses reagents that are much more stable than RIA; and 5) there are fewer manipulations required for ELISA, i.e., ELISA can be run in a single reactive vessel, which thereby increases sensitivity (36).

There are numerous variations of ELISAs detailed in immunology texts and manuals (34,37,38). If classified by methodology, there are three principal types of ELISAs: 1) antigen capture direct assays; 2) antibody capture indirect assays; and 3) two-antibody sandwich assays (34). Assays 1) and 2) will be discussed here as these have been the methods of choice for quantitation of ABA.

The direct assay for ABA, an example of which is produced commercially by Idetek (39), involves the physical and/or chemical attachment of the antibody (monoclonal) to a solid phase, e.g., polystyrene microtiter plate. After appropriate incubation and washing to remove excess antibody, a solution containing a fixed concentration of enzyme-labeled (alkaline phosphatase) ABA and some variable concentration of unknown sample containing ABA is added to the microtiter plate and allowed to incubate. The labeled-ABA and sample ABA will "compete" for the limited antibody sites. Unbound ABA is washed away and the amount of labeled ABA bound to the antibody is measured by the addition of an appropriate

chromogenic substrate, such as *p*-nitro-phenyl phosphate (PNPP) (34,36,39). The measured product concentrations of the enzyme reaction are inversely proportional to the concentrations of the standards and samples.

In the indirect ELISA, ABA conjugated to a large protein, such as bovine serum albumin (BSA), is immobilized onto a solid phase (microtiter plate). An equilibrated solution containing a fixed concentration of antibody (monoclonal) and some variable concentration of unknown sample containing ABA is added to the microtiter plate and allowed to incubate. Excess antibody (not bound to soluble ABA in the sample) will bind "non-competitively" to the immobilized ABA on the microtiter plate, and washing subsequently removes the remaining soluble antibody-sample ABA complex. In the next step, a secondary antibody (raised against the monoclonal antibody) conjugated to an enzyme, or conjugated to biotin for further amplification of sensitivity of the enzyme reaction, is added and allowed to incubate. After washing to remove excess conjugate, an appropriate chromogenic substrate is added, and the resultant colored product is quantified. In the case using a secondary antibody-biotin conjugate to amplify the assay sensitivity, a (strept)avidin-enzyme conjugate is added, allowed to incubate, excess is removed by washing, and the bound enzyme is quantified with an appropriate chromogenic substrate. Again, the measured product concentrations are inversely proportional to the concentrations of the standards or samples.

Although the direct (competitive) ELISA has the advantages of being specific and easy to execute, it does have inherent disadvantages: 1) wasteful use of expensive monoclonal antibody; 2) monoclonal antibodies often bind poorly to plastic microtiter plates thereby

requiring pre-coating of the plates with a polyclonal antibody and increasing the cost of the assay; and 3) possible deactivation of the enzyme-labeled antigen due to incubation with test solutions. The indirect ELISA has the following disadvantages: 1) additional steps which increase the time of the assay and, possibly, the variability of the ABA estimates; and 2) requires additional expensive conjugates, some which may not be commercially available. Advantages of the indirect ELISA include 1) reduction in the amount of monoclonal antibody required per assay (lower cost); 2) ability to amplify using (strept)avidin-biotin in intermediate steps; and 3) as a result of requiring less monoclonal antibody, sensitivity is increased over the direct method. (38,40).

The indirect ELISA was chosen for analyzing ABA in loblolly pine zygotic tissues during seed development. These tissues are extremely small, especially during early embryogenesis. Embryos, megagametophytes, and suspensors required separate analysis, thereby necessitating the most sensitive and specific technique. However, this necessitated development of an indirect ABA ELISA to improve sensitivity and decrease costs over the commercially available assay.

ASSUMPTIONS MADE IN HORMONE ANALYSIS DURING SEED DEVELOPMENT

Before discussing the roles played by ABA during seed and embryo development, the following details must be kept in mind concerning analysis of PGHs in general, and of ABA research during seed development in the past:

1) ABA studies have centered on economically important agricultural species: cereals and legumes, e.g., corn, wheat, soybean, tomato, and barley; several woody angiosperms; but no gymnosperms (prior to this work),

2) bioassays, which tend to represent the composite effects of multiple PGHs, were typically used and, therefore, results using such techniques must be viewed with caution. This is particularly so when the bioassay standards differed from the PGHs isolated from the study tissue, or the tissue used in the bioassay differed from that from which the PGH was isolated,

3) with most PGH analyses, whole fruit (containing seeds) and whole seeds have been assayed as a single tissue as opposed to separate assays for each identifiable tissue type, e.g., embryos, cotyledons, and embryonic axes. Observing PGHs in whole structures (e.g., seeds) often masks changes in concentration in individual seed parts. It also, incorrectly, suggests that PGHs are uniformly distributed throughout heterogenous tissues, and that they have similar functions all the tissues during fruit development. Each tissue and cell type will be influenced differently by each PGH with regard to reaction time and sensitivity (41).

4) correlative evidence, i.e., a proposed role for the PGH based strictly on the simultaneous occurrence of peak values and certain physiological, biochemical, and/or morphological events does not constitute proof of dependence. Causality in correlations between PGH and some growth event can only be inferred and must be substantiated by other types of experiments, such as

application of exogenous PGHs or their inhibitors; or the use of genetic mutants which lack sensitivity to the specific hormone (20,42),

5) the amount of endogenous PGH present has been suggested to govern the magnitude of a developmental response. This premise completely ignores the concept of cellular "sensitivity" to the PGH, i.e., number and affinity of available receptor sites ([glyco]proteins that specifically, and reversibly, bind to specific PGHs), which must also be considered (43). A developmental response should be considered dependent on both the concentration of PGH and the sensitivity of the target tissue towards the PGH (44), and

6) the free hormone extracted at any particular point in time does not necessarily reflect the amount engaged in regulating growth. Extracted free PGH may represent that which:

A) is acting at that site or in the surrounding tissue,

B) is being synthesized at that site for transport to other sites,

C) is being stored at that site to rid other sites of excess PGH, or

D) represents the balance between PGH biosynthesis and "consumption" (45).

ABSCISIC ACID AND ITS ROLE DURING ZYGOTIC EMBRYOGENESIS

The involvement of ABA in the physiology and gene expression of developing seeds and embryos was thoroughly covered in recent reviews over the past eight years (1,10,45,46, 47,48,49). Unquestionably, ABA is the most extensively studied of all the PGHs

playing major roles in embryo and seed development. Although little is known about how ABA operates on a molecular level, two modes are suggested: 1) ABA acts by enhancing the expression of genes that are already active (50); and/or 2) ABA activates genes that normally function at other developmental stages (51). Research studying the changes in endogenous ABA levels during zygotic embryogenesis, application of exogenous ABA to or removal of endogenous ABA from culture, and the use of various ABA mutants have all demonstrated (or strongly suggested) that ABA plays a critical role in seed maturation and development, as well as in the process of germination. Development of genetic mutants (52,53) and analytical techniques (18,54,55) for analyzing ABA function have far surpassed those available for the other PGH in terms of specificity and sensitivity. Routine analytical methods for ABA, such as immunoassays, have reached detection limits in the femtomole (10^{-15}) range, thus permitting analysis of individual seed parts, e.g., embryo, suspensor, and megagametophyte; tissue types, e.g., cotyledons and embryonic axis; and even single cell types, e.g., stoma guard cells (18). Development of these techniques for ABA analysis has accelerated understanding of the physiological roles played by ABA by eliminating reliance on highly variable bioassays (44), such as those still used for many other PGHs.

Although there is a great deal of interest in the effects of ABA on embryo development, there is a paucity of information on the influences of ABA in conifer embryogenesis (reviewed by 10). Economically important species, e.g., cereals, legumes, and a few woody angiosperms have been the species of choice due to their abundant seed availability and large seed size, these providing an abundant and steady supply of experimental tissue, as well as their ease of *in-vitro* culturing to mature plants.

Since its first use in Norway spruce somatic cultures (56), ABA has found its way into all *in-vitro* conifer embryogenic systems (1,57). Unfortunately, very little is known of ABA's physiological, morphological or biochemical function during conifer zygotic embryogenesis. Little more is known of ABA's effects on somatic embryogenesis in conifers, past research tending to be empirical in nature. Fortunately, there are notable similarities in seed and embryo development between well-studied species (angiosperms) and conifers, e.g., ABA stimulates accumulation of storage reserves associated with seed viability after desiccation and dormancy. Thus, it may be possible to draw useful hypotheses as to what role ABA might play in conifer zygotic development from studies of angiosperms. However, one must bear in mind that some acute dissimilarities are known to exist between these two disparate plant groups (10).

ABA Chemical Structure and Characteristics

ABA will be described based on the nine attributes of a PGH (see *Pinus* Zygotic Embryogenesis & Plant Growth Hormones Section):

No. 1 & 2 - ABA has been chemically characterized as an organic substance. It is a C_{15} carboxylic acid (sesquiterpenoid) containing an unsaturated α or β *keto* group (C'_4) and a tertiary hydroxyl group (13), and has been recently proven that ABA is synthesized from mevalonic acid via carotenoid (C_{40}) precursors. (58,59). Naturally occurring ABA is found as a single enantiomer with a single chiral center at the C'_1 carbon and a *cis* double bond at C_2 that readily isomerizes to *trans* in bright light (Fig. 1). The *trans* form is not

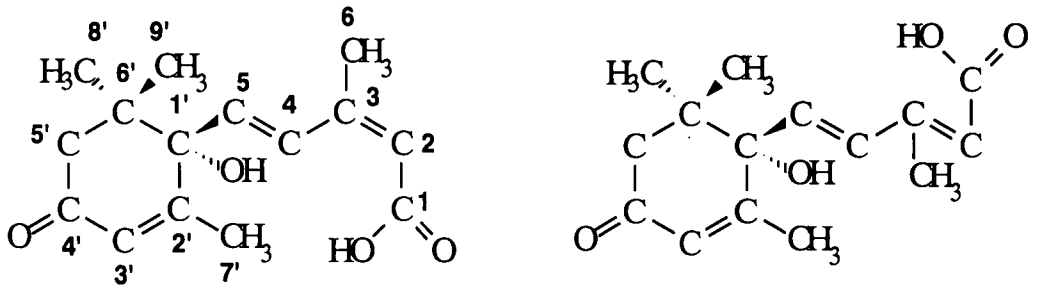

Figure 1. Stereoisomers of ABA: (+)-*cis*-ABA (left) and (+)-*trans*-ABA (right). Carbon numbers are illustrated in the left figure.

thought to be biologically active; what activity has been detected is probably due to its transformation to the *cis* form in ultraviolet light. In addition to stereoisomerism, ABA can also exist as either the (+) or (-) enantiomer, but the (-) form has never been found *in-vivo*. Although there are differing opinions, both enantiomers appear to be equally effective in inhibiting plant growth, however, the (-) form has no effect on stomatal closure (4,13,60) or on promoting maturation of conifer somatic embryos (10). Structural requirements for the biological activity of ABA include: 1) a free carboxylic group, 2) a double bond at the α or β position in the cyclohexane ring, 3) the configuration of the C_2 double bond must be *cis*, and 4) a ketone group in the C'_4 position (60). Chemically, ABA is a weak acid having a pK_a of 4.8. It is chemically stable at low temperature under a wide range of pH conditions: liquid nitrogen to 70°C and pH 2.0-11.0, respectively. Above pH 5.8, ABA is almost completely ionized, and below pH 3.8, ABA exists mainly in an undissociated form (11,13).

No. 3 - ABA is biosynthesized in some plant organ(s). ABA is manufactured in mature leaves, particularly in response to water stress. ABA is also thought to be synthesized in the roots of plants, as well as in the embryos of developing seeds (4,16,61).

No. 4 - ABA is usually translocated from biosynthetic sites to action sites via phloem and xylem tissues. ABA synthesized in the leaves is exported to the phloem tissue where it may circulate to the roots and then return to the shoots via the xylem tissue. ABA may be imported into seed tissues or synthesized *in-situ* (4,42).

No. 5 & 6 - Specific biological activity occurs at extremely low concentrations of ABA, and ABA performs a variety of fundamental roles in regulating biochemical, physiological, or morphological activity *in-vivo*. ABA is known to act as both a growth inhibitor and promotor: stimulating protein synthesis and inducing/maintaining dormancy in developing seeds; and stimulating development of the reproductive buds in short-day plants while inhibiting development in long-day plants. ABA also acts by promoting stomatal closure to reduce transpiration, and accelerating abscission of plant organs and senescence of excised leaf tissues in all species. Exogenously applied ABA exhibits similar biological activities as endogenous ABA in many tissues (8,16).

No. 7 - ABA is broadly distributed throughout plant kingdom. ABA has been detected in many angiosperms and gymnosperms, some ferns, horsetails, and mosses, and in fungi (16,60).

No. 8 - ABA is active in tissues where it is synthesized, as well as in distant tissues. Exogenous ABA has been found effective in causing stomatal closure when applied directly to the stoma or via the xylem tissue (16).

No. 9 - ABA acts in concert with other PGHs. In general, ABA acts as an antagonist to most GAs and some cytokinins in developmental responses. For instance, ABA inhibits α-amylase synthesis and germination in seeds of cereals, but this activity can be counteracted by the application of GAs, and sometimes, cytokinins (16,61). Cytokinins also counteract the accelerated senescence of leaf disks induced by ABA (4,8,16).

Changes in ABA Concentration during Zygotic Embryogenesis

Since the thrust of this research was to determine ABA fluctuations during zygotic embryogenesis in loblolly pine, a review of the literature pertaining to endogenous ABA trends in zygotic tissues is in order. ABA changes during zygotic embryogenesis have not been published for any conifer prior to this work, therefore, this review will be constrained to non-coniferous species.

Changes in ABA concentration during seed and embryo development have been measured in the species shown in Table 1. There are two general trends established for endogenous ABA during zygotic embryogenesis and, fortunately, the majority of these studies have utilized reliable techniques such as GC/MS or immunoassays for estimating ABA levels. The most prevalent trend, by far, in species studied (3,46,48) shows ABA levels that are extremely low or undetectable early in embryogenesis. As development continues, ABA

Table 1. ABA measured in seed tissues throughout zygotic embryogenesis.

Species	Tissues	Reference
Triticum aestivum (wheat)	seed, embryo, endosperm	33,54,62, 63,64,65
Hordeum vulgare L.(barley)	seed, embryo, endosperm	33,65,66
Zea mays L. (maize)	embryo, endosperm, pedicel	67,87
Glycine max (soybean)	pod, seed, seed coat, testa, embryo, cotyledons, embryonic axis	21,41,68, 69,70
Triticale (wheat x rye)	seed	71
Oryza sativa (rice) *Zizania palustris* (wild rice)	embryo, embryonic axis	72
Medicago sativa L.(alfalfa)	embryo, endosperm, testa	73
Pisum sativum (pea)	seed, pod, testa, embryo, endosperm	20,74
Phaseolus vulgaris (bean)	seed, pod, seed coat, embryo, cotyledon, embryonic axis	75,76,77
Phaseolus coccineus (runner bean)	embryo, suspensor, integument	88
Theobroma cacao (cacao)	embryo	78
Lycopersicon esculentum (tomato)	pericarp, seed, testa, embryo, endosperm	25,79,80
Arabidopsis thaliana	seed	81
Helianthus annus (sunflower)	embryo	82
Malus domestica (apple)	embryonic axis	89
Pyrus communis L. (pear)	seed	84
Prunes Persia (peach)	embryo, endosperm, nucellus, integuments	90
Ace platanoides L. (Norway maple)	embryo	91

levels increase reaching one or more maxima at middle to late embryogenesis (time from embryo initiation to seed maturity), and then drop to a low level, typically coinciding with cessation of dry matter accumulation and accompanying or preceding desiccation. This trend is illustrated in Figs. 2A and 2B for tomato (25) and wheat (64), respectively. The peaking of ABA levels generally occurs when GA and auxin have decreased to their lowest levels (20). The late drop in ABA may be attributed to metabolic degradation (16,86) and/or conversion to conjugated storage forms (16,75,85).

The second most observed ABA trend is one in which ABA is extremely low or undetectable early in embryogenesis, similar to the above trend except that ABA levels continue to rise until seed maturity (87,88,89,90), or drops slightly just prior to seed maturity (74,87,91). This trend is illustrated in Fig. 2C for Norway maple (91). With the exception of peach, woody angiosperms, i.e., apple and Norway maple, fall within this category.

There were no apparent trends with regards to which tissue contained the most endogenous ABA. In alfalfa (73), ABA in endosperm tissues was greater than in embryo tissues, whereas in maize (67), the opposite was true. In a few studies, "bound" forms of ABA (proposed storage forms of ABA), i.e., ABA covalently bonded to glucose as either the *D*-glucosyl ester or the C'_1-glucoside (13,92) were also measured in fruit, embryo, and grain tissues. "Bound" ABAs generally paralleled free ABA trends during zygotic embryogenesis and were typically present in much lower concentrations than free ABA (1-14%) (16,25,49,62), but in some cases equalled or exceeded free ABA levels (25,89).

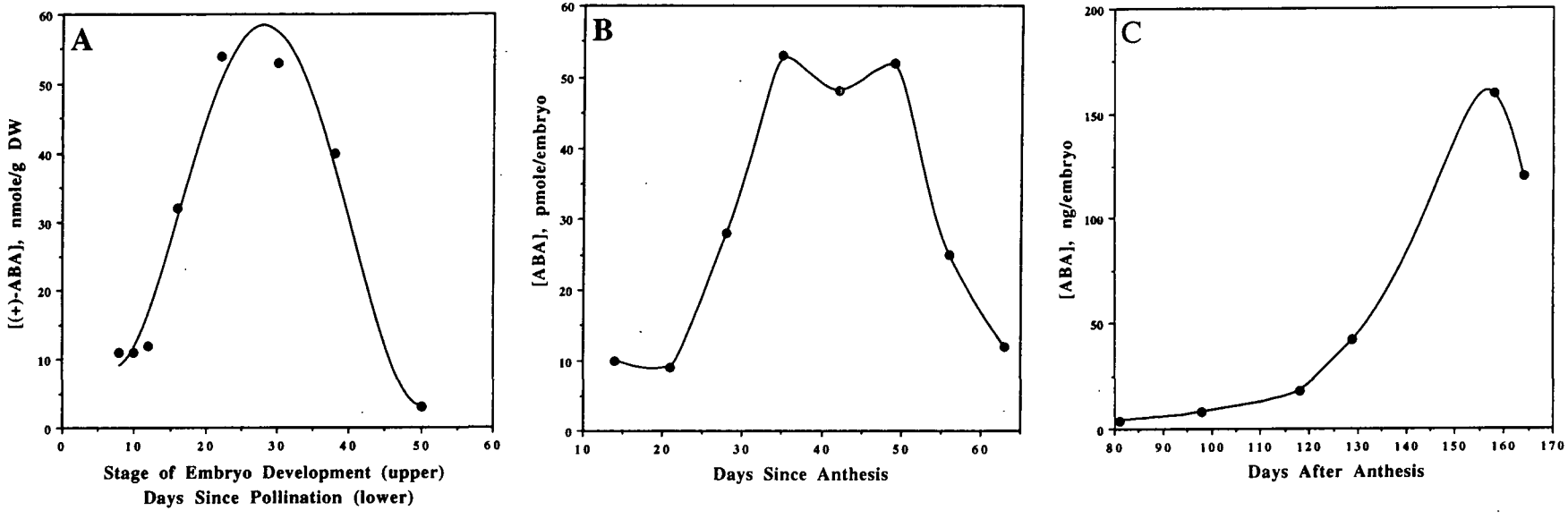

Figure 2. Endogenous ABA concentrations during zygotic embryogenesis. **(A)** Tomato embryos (25). **(B)** Wheat grains (64). **(C)** Norway maple embryos (91).

Basis for Concentration/Content Units for ABA

Before discussing putative roles of ABA during seed and embryo development, the concentration units in which ABA was measured in plant tissues warrants comment. Jones and Brenner (67) and Rajasekaran et al. (93) address this issue in great detail. ABA can be expressed on a concentration (dry-, fresh-, or water-weight) or on a content (per organ, e.g., embryo, endosperm, seed, etc.) basis. Each of these methods has advantages and disadvantages. Representing ABA on a dry- or fresh-weight basis can be useful in reducing the variability between ABA measurements with tissues of similar stages, but different sizes. However, this method can result in underestimation of ABA content in tissues that are drastically changing in weight, e.g., seeds accumulating reserve stores. These bases also falsely presume that ABA is uniformly distributed throughout heterogeneous tissues. Although expressing ABA on a water basis assumes homogeneous distribution throughout the available water in the tissue, it does correct for growth and the accumulation of reserve proteins. If tissues are very small, the error involved in weighing can strongly affect the accuracy of the estimates.

Determination of ABA on a content basis (per organ) does not consider increases in tissue mass during development, but it can be more meaningful than concentration for relating to the physiological effects of ABA. It also does not imply uniform distribution of ABA in heterogeneous tissues such as those which make up the embryo.

From this discussion, no one method is superior and valuable information can be obtained from each. Dry- and fresh-weight concentration and organ bases were most frequently used in the literature.

Time Scales Used when Determining Trends During Zygotic Embryogenesis

By far the most widely used scale for measuring PGH trends during zygotic embryogenesis has been a linear time scale: days since fertilization (DSF), pollination, anthesis, or flowering; in a few cases, a developmental scale representing changes in embryo morphology was utilized (73,63,88); but only rarely were both used (25). The popularity of using a linear time scale is its ease for collating data by calendar date and in visualizing PGH changes over the growth period. The disadvantages are that embryo developmental rates are not identical within or between plant species or even within the seed pod of one plant and, therefore, this scale would not correlate PGH changes with development of tissue features. Also, since embryo development occurs at different rates in different species, it is difficult to compare trends between species on a linear time scale as there is no common frame of reference, e.g., embryo morphology.

Measuring PGHs trends on a embryo morphology basis is more difficult as the embryos must be visually "staged" during collection. This procedure can be tedious and prone to subjective bias if proper precautions are not exercised. Advantages are that the results are more useful in comparisons between species or, e.g., in comparing zygotic versus somatic development. The disadvantage of collecting tissue strictly on the basis of developmental stage is that it might mask trends based on linear time (developmental stages may last

from several days to weeks). Using both linear time and embryo development on a single axis can give all the advantages with few disadvantages.

Causal Roles for ABA During Seed Development

Determining the role of ABA based on its concurrent appearance (correlative evidence) with some physiological, biochemical, or morphological event has lead to few tangible conclusions, many contradictions, and a great deal of confusion as to the true role of ABA. It is surprising that researchers have continued to use this dubious method, without collaborative evidence, for determining the roles of PGHs for over the past 25 years. Causality between PGH fluctuations and some growth feature can only be inferred and must be validated by other types of experiments, such as application of exogenous ABA, use of ABA promoters and/or inhibitors to alter endogenous levels, or identification of genetic mutants which are deficient in hormonal biosynthesis or sensitivity. Keeping this in mind, endogenous ABA has yet been implicated as a controlling factor for many processes during *in-vivo* seed and embryo development.

In the embryos (41,68) and seeds (21) of soybean, ABA accumulated rapidly at the time when cell division, DNA and RNA synthesis, and fresh- and dry-weight were greatest. This suggested that ABA was involved in the onset of increased growth rate in seeds and embryos. A decrease in the rate of dry weight accumulation of the seed occurred concurrently with a sharp drop in ABA levels. A causal relationship between maximum growth rates and dry-weight accumulation and maximum ABA concentrations has been also suggested in the grains of wheat (54,62,63), barley (66), and maize (67), in the seeds and

embryos of tomato (25,80), rice (72), bean (75,76), pea (74), sunflower (80), pear (84), and peach (90), and in integuments and embryos of runner bean (88). It has been suggested that ABA acts by either by promoting protein synthesis (68) and/or stimulating phloem unloading (41,45,94), although Schussler et al. (70) later refuted this latter claim in soybean (41). Figure 3 illustrates the proposed causal relationship between endogenous ABA and dry weight accumulation in the kernels of *Triticale* (71). The reader should be aware that all the established PGHs have been "causally" implicated as playing a critical role in growth rate and dry-weight accumulation as their peak concentrations have also coincided with these physiological events (20,95,96).

In conflicting studies with wheat and barley, Quarrie et al. (33) found that there was no causal relationship between ABA levels and grain filling, maximum grain growth rate, or in the final grain weight. Morris (64) found in wheat that synthesis of germ agglutinin during late development was not correlated to ABA concentration, but suggested that a change in ABA sensitivity by the embryo might be responsible. Schussler et al. (70) found in soybean that seed growth rate was not associated with endogenous ABA and that sucrose levels, which resulted in an increase in fresh-weight, were also not closely coupled to the concentration of ABA. Therefore, it is not possible to deduce the role of ABA in controlling growth rate or dry-weight accumulation from correlative evidence alone.

Researchers have also suggested that the accumulation of endogenous ABA during late stages of development prevents the embryo from advancing directly from embryogenesis

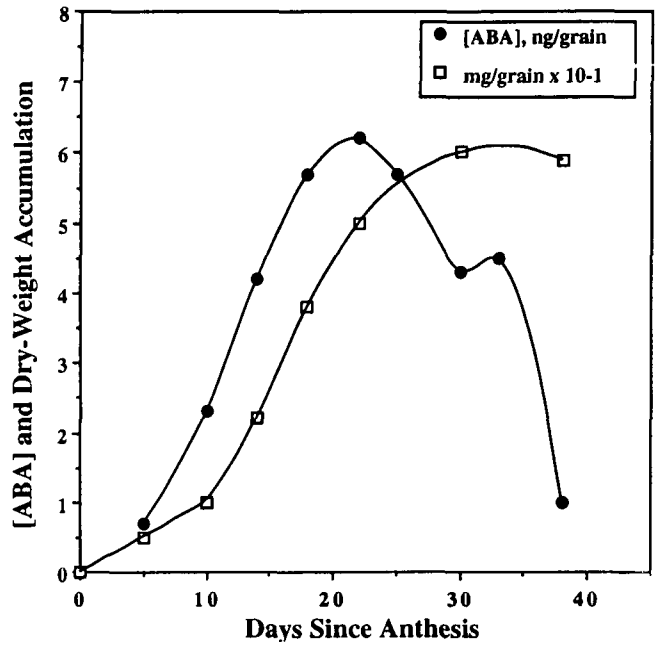

Figure 3. Causal relationship between endogenous ABA and dry-weight accumulation in *Triticale* kernels (71).

to germination, i.e., ABA inhibits precocious germination, thereby allowing the seed time to mature and accumulate storage reserves for normal germination (41,49,69,70). In wheat, germination of immature grains increased from 20 to 25 days after anthesis (DAA), but this was subsequently followed by a 15 day period of natural dormancy which corresponded to the highest levels of endogenous ABA. Full germination was reattained with a decrease in ABA and rapid water loss at 40-60 DAA (62). In pea, a significant increase in germination capacity was associated with a rapid decline in ABA concentration (20) (Fig. 4), high levels of ABA have been associated with induced dormancy in the seeds of bean (76). In the case of bean, germination was not possible until endogenous ABA levels began decreasing, and as ABA levels decreased, the lag time for germination shortened. High levels of endogenous ABA has also been correlated with the suppression of precocious germination in immature

embryos of wheat (63), bean (77), apple (89), and Norway maple (91). Le Page-Degivry et al. (82) found immature sunflower embryos would germinate when ABA levels were low, but that dormancy was established immediately after endogenous ABA levels peaked and dropped. They concluded that dormancy might be dependent on ABA synthesis, but not concomitant with its accumulation. It was also possible that sensitivity to ABA was greater during the later stages of development when ABA levels and germination were low (82,97) and that immature embryos must advance to a certain stage of development before ABA has any affect (speculation).

Although falling outside the topic of discussion, i.e., embryogenesis to seed maturity, there are two studies worth mentioning as they involve measuring ABA trends during conifer seed stratification and germination. Murphy and Noland (98) studied ABA trends during stratification in *Pinus lambertiana* Dougl. (sugar pine) embryos and mega-gametophytes. Levels of endogenous embryonic ABA were found to be relatively high at the onset of stratification and remained unchanged for the first 30 days while germination increased from 5 to 20%. ABA levels subsequently decreased by 62% from day 30 to day 90. Megagametophytic ABA remained constant during stratification at about 5% of embryonic ABA levels. They concluded that loss of seed dormancy in sugar pine was not correlated to the change in ABA, but instead possibly due to increased levels of growth promoters (none specifically mentioned) or a change in metabolic pathways. The authors did not speculate on whether a change in ABA sensitivity might explain the increase in germination frequency during the first 30 days when ABA levels remained constant.

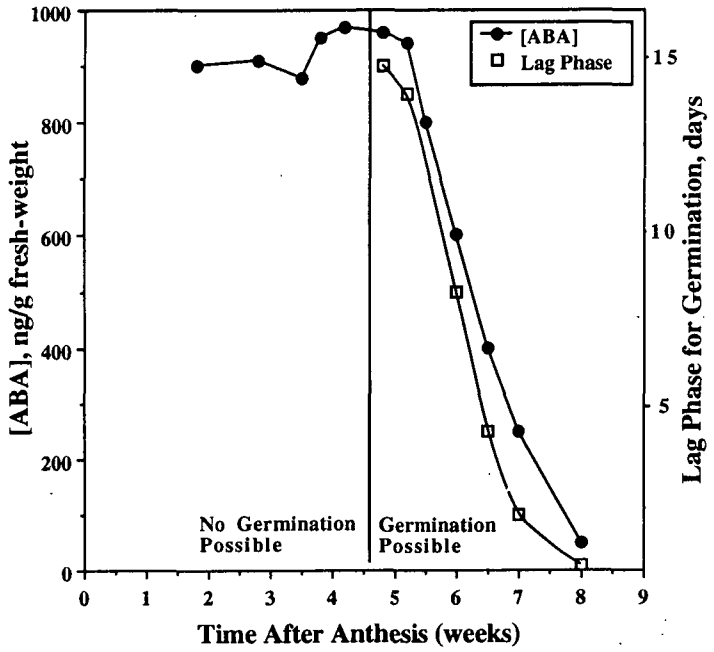

Figure 4. Causal relationship between endogenous ABA and lag phase for germination in bean seeds (76).

Paul et al. (99) used bioassays to quantitate changes in growth "inhibitor" (presumably ABA) and "promotor" (GA-like) substances during cold stratification and germination in the seeds of *Pinus taeda* (loblolly pine). Their results indicated that unstratified and cold-stratified seeds up to day 28 contained high levels of an "inhibitor" and little to no growth promoting substances. After 42 days of stratification, "inhibitor" levels dropped to almost zero as the "promotor" levels gradually increased. Germinating seeds had very high levels of the growth "promotor" and no "inhibitor". They suggested that the lack of growth "promoters" and/or the presence of the "inhibitor" might be the cause of dormancy in loblolly pine and that the long-term cold stratification somehow reduced the "inhibitor", and thereby allowed germination.

Endogenous ABA has been implicated in the control of desiccation tolerance in several species including soybean (21,68), tomato (25), wheat (62), *Triticale* (71), and bean (75). This was mainly due to the fact the water content of seed tissues was rapidly decreasing at approximately the same time as ABA levels dropped. Figure 5 illustrates the causal relationship found between endogenous ABA and water content in the kernels of *Triticale* (71).

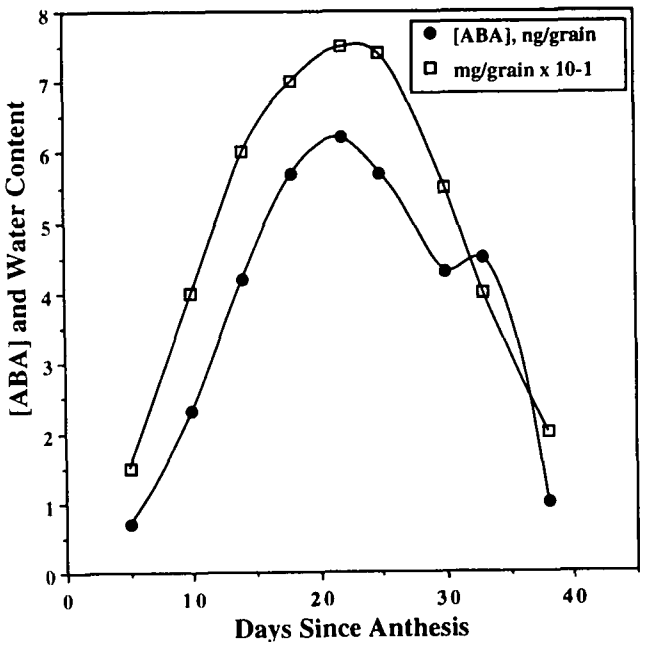

Figure 5. Causal relationship between endogenous ABA and water content in *Triticale* kernels (71).

ABA has also been associated with the suppression of premature reserve mobilization. King reported that in *Triticale* (71) high levels of ABA during the late stages of development occurred concomitant with low α-amylase activity and that enzyme activity

increased dramatically when ABA levels dropped (Fig. 6). Addition of GA to wheat grains only triggered α-amylase production when ABA levels were dropping.

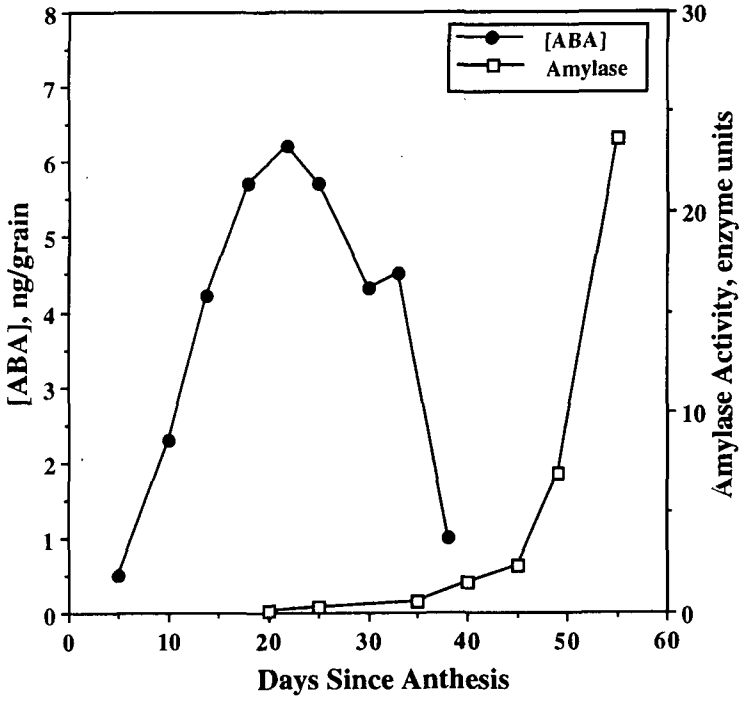

Figure 6. Causal relationship between endogenous ABA and amylase release in *Triticale* kernels (71).

Changes in ABA Concentration During Somatic Embryogenesis

Fluctuations of ABA during somatic embryogenesis warrants review since one objective of this thesis was to obtain information for use in altering ABA levels in somatic embryogenesis to mimic those measured in zygotic embryogenesis. There are very few published reports on the fluctuations of endogenous ABA during somatic embryogenesis, and none for trends during conifer somatic embryogenesis. Trends were measured in somatic embryos of *Vitis vinifera* L x V. *rupestris* Scheele (gloryvine) (93) and *Daucus carota* L. (wild carrot) (100), as well as in cell suspensions of *Brassica napus* L. cv. Kasan (rapeseed)

(101) and *Oryza sativa* L. cv. Bahia (rice) (102) (exogenous ABA was added to the culture media of gloryvine). In gloryvine, wild carrot and rapeseed, endogenous ABA trends were similar to those found in most zygotic systems; a rapid rise in ABA mid-to-late in development as shown in Fig. 7 for gloryvine.

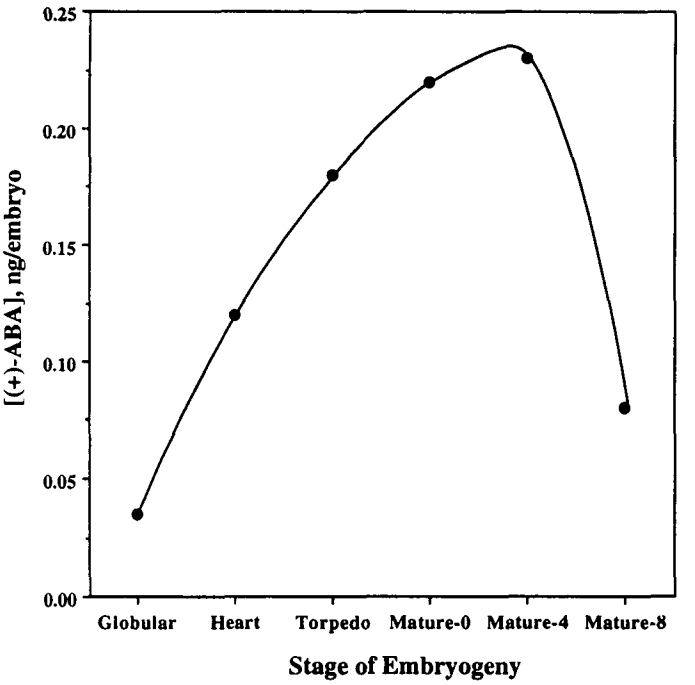

Figure 7. Endogenous ABA levels in developing somatic embryos of gloryvine (93). Numbers after mature indicate weeks. Mature-8 are fully developed seeds.

In unchilled somatic embryos of gloryvine (93), ABA levels increased rapidly on a per embryo basis from the globular (0.035 ng) to the mature stage (0.22 ng), and then decreased very rapidly to original levels (0.08 ng) 8 weeks after maturity (Fig. 7). On a dry weight basis, ABA levels were highest at the globular stage and decreased to the late mature (8 weeks) stage. This decline in ABA level must be due to the rate of dry-matter accumulation between the globular and mature stage because the ABA on an embryo basis remained relatively unchanged during most of development.

Chilling (4°C) of mature gloryvine (93) somatic embryos led to a significant reduction in endogenous ABA and was required for normal germination of mature embryos. Chilling of globular, heart, and torpedo-shaped somatic embryos also led to a significant reduction in ABA, but resulted in precocious germination. Thus, dormancy appeared to be associated with high levels of ABA, and chilling lead to a reduction of ABA which, in turn, broke dormancy and promoted germination. Moreover, addition of exogenous ABA inhibited germination of chilled embryos.

A rapid rise in ABA induced artificial dormancy in wild carrot embryos and was concurrent with a reduction in embryo abnormalities (100). Rising ABA levels were also correlated with rising osmotic potential in rapeseed embryo cell sap, possibly explained by a restricted uptake of sucrose, glucose, and fructose (101).

In rice cell suspension cultures, ABA remained constant until the stationary phase when levels increased. It was suggested that ABA played a role in the inhibition of cell culture growth by initiating senescence of the cells (102).

Physiological Effects of ABA in Culture and the Use of ABA Mutants

Exogenous application of ABA *in-vitro*, use of ABA analogs, washing or slow drying to control endogenous levels of ABA, and use of ABA mutants that synthesize low levels of the hormone or possess varying sensitivities to ABA, can all provide further evidence to support roles for ABA in seed development. Possible functions for ABA during seed development have been thoroughly reviewed by Black (46) and Quatrano (48) and an

overview of putative roles for ABA in this process will now be discussed. Increasingly, ABA is being thought of as a "stress" hormone rather than a strict "inhibitor". ABA acts, sometimes in concert with osmotica (water stress), to prepare the seed for survival during adverse climatic conditions by promoting physiological functions that increase the seeds chances for avoiding death, i.e., embryo maturation, accumulation of protein reserves, desiccation tolerance, and dormancy. Recent studies show that ABA effects during embryogenesis (including inhibition of precocious germination, dormancy, reserve protein accumulation, and desiccation tolerance) are either developmentally controlled or stress induced (10).

Precocious Germination

Isolation of immature embryos from developing seeds liberates them from their normal course of development, thus, allowing premature germination to occur. A result of this early germination is aberrant, overly-elongated cotyledons, poor hypocotyl development, low root emergence, and almost no storage protein accumulation which typically results in death (103). The addition of ABA to the culture medium will commonly suppress precocious germination and allow immature embryos to mature naturally, and in many instances, accumulate storage proteins. Black (46) concluded that, "embryos *in planta* are held in embryogenetic growth by native ABA; (and) when isolated, they lose the ABA, and germinative growth is permitted, but if the ABA content is maintained, embryogenetic growth continues." This conclusion is supported by correlative evidence that suggests that high

endogenous ABA concentrations may act as a trigger in suppressing precocious germination during zygotic embryogenesis.

The use of chemical inhibitors and promoters of ABA action, washing or slowly drying embryos, and utilization of genetic mutants have also demonstrated the role of ABA in suppressing precocious germination. Fluridone (1-methyl-3-phenyl-5-(3[trifluoromethyl]-phenyl)-4-(1H) pyridone), a non-specific pyridinone inhibitor of carotenoid biosynthesis (precursors to ABA) (82,104), reduces endogenous levels of ABA in tissues. Embryos (in seeds) incubated with fluridone will germinate precociously. The addition of ABA to these cultures re-institutes suppression of precocious germination. But, as fluridone can also affect other metabolic processes (105), results obtained from these types of studies must be considered with some skepticism. In soybean, washing isolated embryos, or allowing them to slowly dry (in-pod or isolated), resulted in a decrease in endogenous ABA and induced precocious germination (69).

Probably the most reliable means of ascertaining the role of ABA during seed development would be through the use of genetic mutants. Mutants with low capacities for ABA biosynthesis (ABA-*deficient*, e.g., ABA levels ≤ 10% of wild type) and mutants with low sensitivities to ABA (ABA-*insensitive*) exhibit "vivipary" (precocious germination of embryos while the seed is still attached to the mother plant). In these cases, precocious germination occurred either because too little endogenous ABA is produced or because the embryos simply do not respond to the ABA present (46). In immature wild-type and *vp5/vp-*

5 (ABA-*deficient*) embryos of maize, *in-vitro* culture with ABA prevented precocious germination (52).

Such precocious germination can also be arrested by the addition of osmotica in culture (in the absence of ABA) suggesting that ABA and osmotica are correlated in some fashion. Low osmotic conditions, which generally do not elevate ABA levels, encourage immature embryos to remain in a developmental mode and mature normally, thus mimicking the presence of ABA. Isolated immature wild-type and *vp-5/vp-5* (ABA-*deficient*) embryos of maize cultured with mannitol (osmotic stress) did not germinate precociously. In the case of immature *vp-1/vp-1* (ABA-*insensitive*) embryos, only osmotica suppressed premature germination (52). Therefore, ABA and osmotica (in the absence of ABA) both suppress premature germination probably by acting through different mechanisms since the embryos did exhibit differences in germination rates, growth rates, storage protein accumulation, or metabolism (46,52). It has been suggested in the past that ABA affects water uptake into the seed or embryo by retarding cell-wall loosening and thereby increasing the minimum turgor threshold required for germination. Osmotica reduces seed turgor by reducing seed water content (46,97,106).

Dormancy

The induction of dormancy is a critical stage of seed development which enables a plant species to survive periods of severe environmental stress. Dormancy is most likely caused by internal factors, e.g., hormonal status or energy change. As most findings to date have been contradictory, the extent to which ABA induces and/or maintains dormancy is still

in debate. Current research implicates ABA in initiating dormancy, but not its maintenance (46). In most species, there does not appear to be causal relationship between dormancy and endogenous ABA content. For instance, Walker-Simmons (54) found that in high- and low-dormancy wheat varieties, embryonic ABA content was not significantly different, but in a later study with the same wheat varieties she found that, although dormancy was not associated with the absolute ABA content, it was correlated with a prolonged elevation of ABA during maturation (63).

Contrary to Walker-Simmons work, research with maize suggested that ABA might only act as a trigger to dormancy and that the continued presence of ABA is not required since very little was found in the mature grain (46,67,82). In wheat, soybean, and rapeseed, as embryonic ABA levels declined, there was a concurrent drop in tissue sensitivity to ABA suggesting that some factor other than ABA, possibly desiccation, was responsible for inhibiting germination late in development (48). Conflicting with this finding was evidence which suggested that ABA may control water uptake in seeds, possibly hindering the ability of embryos to absorb water, and thereby inducing dormancy (107). In soybean, removal of ABA from the embryos, either by washing or drying, resulted in germination, and the degree of the germination was related to the length of washing or drying (48). Washing achenes from *Rosa* (rose) in water + charcoal reduced the endogenous ABA concentration (ABA was detected in wash water) and increased the germination percentage (108).

In some species, there is convincing evidence suggesting that ABA acts on a genetic level. For example, ABA inhibits the production of specific germination enzymes:

carboxy-peptidase C in cotton embryos, ribulose bisphosphate carboxylase in wheat embryos, and isocitrate lyase in castor bean embryos (48). Findings also suggest that ABA may participate in dormancy by inducing expression of gene products that participate in the induction of dormancy, i.e., "dormancy proteins".

In wild-type *Arabidopsis* (normal ABA levels), dormancy occurred during maturation, but in the ABA-*deficient* (*aba*) mutant, dormancy did not occur at all. In the ABA-*insensitive* (*abi*3) mutant, dormancy was reduced, but was still exhibited to some degree. In two newly isolated ABA-*"severely" insensitive* mutants (*abi*3-4 and *abi*3-5), dormancy was even further reduced. The amount of exogenous ABA required to obtain total dormancy was opposite of this trend, and even extremely high levels of ABA were not capable of completely suppressing germination in *abi*3-4 and *abi*3-5 (53). Studies with *Arabidopsis* and tomato also showed that it was mainly embryonic ABA that was required for dormancy. However, maternal ABA contributed to a lesser degree, while exogenous ABA could not induce dormancy at all (46,48).

Since Blacks' review (46), additional work has further clarified the role of ABA in dormancy. Using ABA and GA mutants in tomato, it was determined that seed dormancy and germination rates were controlled primarily by critical shifts in embryonic water potential thresholds, possibly as a result of water accumulation or loss of solutes in the embryo. These thresholds occurred mainly as a result of cell wall hydrolase activity which lead to endosperm weakening and radicle emergence. Findings indicated that these thresholds were regulated by ABA and GA levels. Low levels of ABA in the ABA-*deficient* seeds resulted in very low

water potential thresholds and no dormancy. Incubation of seeds with ABA increased the water potential and induced dormancy. In the GA-*deficient* seeds, the water potential for germination was above 0 MPa and no germination occurred even in water. Incubation with GA_{4+7} caused a shift in water potential to lower levels, resulting in higher germination rates and shorter times to germination (107).

Subbaiah and Powell (109) studied the endogenous ABA levels during warm and cold stratification in *Malus domestica* (apple), a species requiring chilling for germination. They found that the embryonic ABA content during both warm (20°C) and cold (5°C) stratification remained constant, but only cold stratified seed would germinate. Their conclusion was that the length of the chilling period for apple seeds was not associated with embryonic ABA content. ABA content in the nucellus and seed coat (essentially dead tissues) dropped dramatically and then remained constant during stratification. Analysis of leachate (deionized water) found levels of ABA corresponding to the losses from these tissues. This work could support the conclusion that ABA is not required for maintaining dormancy in apple.

In sugar pine, Murphy and Noland (98) concluded that ABA was not critical for maintaining dormancy during stratification. Soaking 90 day old stratified and de-coated seeds in ABA increased the germination rate by only 9%. They concluded that the loss of seed dormancy in sugar pine during stratification did not appear to be the result of ABA loss from the embryo or megagametophyte. There was no mention of the possibility of changing tissue sensitivity towards ABA during stratification.

Reserve Products

Past research suggests that ABA is directly involved in the regulation of reserve accumulation in embryos and endosperm of developing seeds (46). In isolated cotyledons or immature zygotic embryos of soybean, maize, and *Vicia faba* (46), as well as in alfalfa somatic embryos (110), the addition of ABA to *in-vitro* culture, at levels which inhibit precocious germination, promoted reserve protein accumulation and resulted in an increase in fresh- and dry-weight. It should be noted, however, that age did play a role in determining the responsiveness of these tissues towards ABA. ABA effects on non-protein reserves, such as triacylglycerol and fatty acid composition, have also been studied in *Brassica napus* (canola) (46), *Apium graveolens* L. (celery) (111), and wheat (112) with similar results. Additional support for ABA's role in reserve accumulation emerges from work with ABA mutants. Seeds from *Arabidopsis* ABA-*deficient* x ABA-*unresponsive* mutants did not produce certain storage proteins found in the wild-type variety (normal ABA levels and sensitivities) (46,48). In immature, as well as mature embryos from *viviparous* maize (ABA-*deficient*) mutants, a specific level of endogenous ABA was required during maturation for the expression of the *Globulin* gene which encodes the two most abundant storage globulins, *Glb1* and *Glb2* (87,113).

On the other hand, in *Pisum sativum* (pea), backcrossing a ABA-*deficient* mutant with wild-type pea (to obtain pods with both ABA-normal and ABA-*deficient* seeds) resulted in no differences in the seed growth patterns suggesting, that ABA did not perform a critical role in pea seed growth (114). There was no mention as to whether ABA deficiency is a

dominant or recessive trait. Similarly, in ABA-*deficient* tomato (80), very low levels of endogenous ABA did not change the final dry- or fresh-weights of the mutant seeds, nor the accumulation and composition of the storage proteins. Thus, although correlative evidence in most species supports an association of ABA with reserve accumulation as high levels of ABA are generally concurrent with maximum fresh- and dry-weight accumulation, this is also true for IAA (95), GAs (20), and cytokinins (96) leading to skepticism of the correlative evidence.

The proteins affected by ABA are of various types, e.g., reserve-types (e.g., legumin, napin, vicilin, wheat germ agglutinin, etc.); proteins present during early embryogenesis; proteins destined for chloroplast import; and *LEA* (*L*ate-*E*mbryogenesis *A*bundant) proteins. All of these proteins appear to be controlled transcriptionally (mRNA) (115). The addition of ABA to isolated immature embryos (to suppress precocious germination) typically induces the premature accumulation of *LEA* proteins through action at a genetic level, i.e., promoting transcription from the *LEA* genes. This is supported by correlative evidence in zygotic embryogenesis where high levels of ABA are associated with the presence of *LEA* mRNA late in embryogenesis (46,48). *LEA* mRNAs and proteins persist in the dry seed (47) and are quickly metabolized within 36 hours after germination when ABA levels are dropping (48,115).

However, even in this respect, not all research has been supportive. In canola, the application of exogenous ABA to haploid embryos in culture did not result in high transcript

levels from *LEA* genes. *LEA* mRNAs have been also induced in germinating seedlings of several species after incubation with ABA (116).

As with precocious germination, osmotica (water stress) has been found to play an active role in the regulation of protein accumulation, apparently operating at a genetic level similar to ABA (46,112). In general, high osmotica (water stress) promotes the deposition of reserve and *LEA* proteins in culture without raising ABA levels and, in some cases, even more effectively than ABA. In immature *vp-1/vp-1* (ABA-*insensitive*) embryos of maize, *in-vitro* culture with ABA produced mRNAs that encode a polypeptide of 30 kDa, while culture with mannitol (osmotica) produced mRNAs that encode a polypeptide of 30 kDa, as well as a set of 20 kDa polypeptides. These results suggest two independent pathways for ABA and osmotic stress (52): one pathway is controlled through an increase in the level of ABA, and the other mediated via an osmotic pathway (10).

Research also suggested that the appearance of reserve protein genes occurred when germinative cell elongation was obstructed (i.e., inhibited precocious germination) either when using osmotica, by reducing water potential, or when using ABA, by restricting cell-wall extensibility. In both cases, the embryos continued to develop normally. For ABA, this would imply that gene expression could be controlled by a limitation in water, and not as a direct result of ABA levels (46).

The above review refers only to the role of ABA during the late stages of embryogenesis. Myers et al. (117) studied the effects of ABA during early embryogenesis in isolated maize kernels. They found that elevated levels of exogenous ABA during early

development (5-10 DSP) decreased the rate of cell division in the endosperm and possibly limited the final kernel weight. Endosperm expansion appeared unaffected, and nuclear size, as well as starch granule number, appeared only slightly negatively affected. Overall, the final embryo dry weight was unaffected and the results demonstrated the importance of developmental age of the tissue with respect to the affects of ABA, i.e., the embryo may need to advance to a certain stage of development before ABA has any effect.

Desiccation Tolerance

At some point in seed development and maturation, seed tissues in most species begin to dehydrate. This process is critical, not only in modulating the progression of development from maturation to germination (48), but also for the long term survival of the seed after it has detached from the mother plant (118). At some stage of development, the seed tissues must become desiccation tolerant or lose viability. Tolerance to desiccation is defined as the capacity of a seed to germinate after short-term storage (48 hours) in a dry state (53).

Tolerance to drying and maximum conversion to seedlings of alfalfa somatic embryos was enhanced by exposure to ABA during maturation. Certain sublethal stress treatments, e.g., chilling, osmotica (via water stressing or sucrose), and especially heat shock, were also effective in inducing desiccation tolerance, by increasing endogenous levels of ABA (110,119,120). Since desiccation tolerance in many species is acquired concurrently with high levels of ABA during zygotic embryogenesis, ABA has been implicated in conveying this tolerance. Various carbohydrates, proteins, and polypeptides (including *LEA*) accumulate

during late development, presumably through the action of ABA, and some of these compounds are also implicated in conveying desiccation tolerance (46,121) (although not all research supports this conclusion). Some of these compounds have unique characteristics that might enable them to protect membranes from dehydration effects, as well as maintaining enzymatic activity (115, 122). Desiccation tolerant species have also been shown to form a "glass" (i.e., highly viscous liquid) from a variety of available oligosaccharides during dehydration (<10% moisture), an ability not found in intolerant species. This glass may function in safeguarding membrane structures during desiccation (123). Accumulation of disaccharides could enhance tolerance by interacting with the polar groups of phospholipids and the functional groups of proteins, or by replacing the hydration layer surrounding membranes, thereby preventing structural damage as water is removed (124). Sugars could also facilitate vitrification, i.e., process of making a glass, thereby avoiding damage caused by crystallization during drying (125).

Increased sucrose accumulation has been associated with high levels of endogenous ABA (*in-vivo*), as well as exogenous ABA (*in-vitro*), in soybean embryos (45). ABA has also been implicated in stimulating sugar mobility in sugar beet, grape, pea, and bean (ref. in 70), but not in bean embryos (44). However, recent work with developing embryos and seed coats of soybean (70), as well as cell suspensions of *Brassica napus* L cv. Kasan (oilseed rape), treated with the non-specific ABA biosynthesis promotor BAS 111..W (which also hampers GA biosynthesis) (126), has not supported a role for ABA in the regulation of sugar mobility (101).

Research has demonstrated that embryos formed by the double mutant strain *aba,abi3* obtained from crossing ABA-*deficient* (*aba*) and ABA-*insensitive* (*abi*3) mutant of *Arabidopsis thaliana* retained a high moisture content during development, were desiccation intolerant, and failed to produce the *LEA* proteins found naturally in wild-type embryos (normal ABA synthesis and sensitivity) (46,48). This has also been recently supported by Meurs et al. (127) who discovered that the double mutant seeds, which were unable to synthesize low molecular weight polypeptides (similar to *LEA*) and storage proteins (whose possible role might be as protective substances), rapidly lost viability when dried. Treatments with ABA, or ABA + sucrose prior to drying restored protein accumulation and allowed up to 100% germination.

Ooms et al. (53) isolated two new *Arabidopsis* mutants that were *"severely" insensitive* to ABA: *abi*3-4, and *abi*3-5 (both much more *insensitive* than the double mutant *aba,abi3*). During the course of seed development, wild-type (normal ABA) seeds were the first to become desiccation tolerant, followed by *abi*3, *abi*3-4, and then *abi*3-5. Analysis of carbohydrates showed that the desiccation tolerant wild type and *abi*3 had little accumulated soluble sugar (sucrose), but high levels of oligosaccharides (raffinose and stachyose) late in seed development. This suggested a relationship between a low mono-/oligosaccharides ratio ("glass" formation premise) and desiccation tolerance, but not total accumulation of carbohydrates or synthesis of a few ABA-*responsive* proteins. Also, accumulation of ABA-responsive "maturation proteins" alone was not sufficient for inducing desiccation tolerance in isolated soybean axes (46,53).

Recalcitrant seeds, i.e., seeds that can not tolerate desiccation even when mature, also appear unable to produce *LEA* proteins (although it is not known if ABA is involved) (46). ABA-*deficient* and ABA-*insensitive* mutants of maize do not dehydrate, nor do they synthesize *LEA* products. Still et al. (72) studied dehydrin proteins (hypothetical subset of *LEA* proteins), ABA, and the development of desiccation tolerance in paddy rice (*Oryza sativa*) and *mildly* recalcitrant (*Zizania palustris*) wild rice (different genus). Embryonic ABA concentration in wild rice was more than twice that found in paddy rice, suggesting that ABA was not responsible for the desiccation intolerance of wild rice. There was also no apparent relationship between ABA concentration and expression of dehydrin protein in either species and, although protein synthesis began before desiccation tolerance had developed, the major accumulation occurred after most of the seeds in either species were tolerant. As the sucrose level increased, so did the level of tolerance in both species, but the levels of sucrose in wild rice were almost twice as high as in paddy rice, suggesting that something other than sucrose was important in determination of desiccation tolerance.

As with many of the putative roles for ABA, osmotica have been shown to mimic ABA's effects on desiccation tolerance. In soybean, it was found that "maturation" proteins (*LEA*) accumulated late in embryogenesis concurrent with the induction of desiccation tolerance. During imbibition, either exogenous ABA or polyethylene glycol delayed the disappearance of the *LEA* proteins, loss of desiccation tolerance, and germination. They concluded that these maturation proteins contributed to, but did not induce, desiccation tolerance (121).

Although research in the past has strongly endorsed the role of ABA in desiccation tolerance, work since Blacks' review (46) has both supported and opposed this function. In *Apium graveolens* L. (celery), desiccation tolerance was enhanced by incubation with ABA or proline during maturation and the combination of the two gave the greatest tolerance and accumulation of fatty acids. This suggested that ABA might bestow desiccation tolerance by increasing the fatty acid content, an affect known to improve maturation and desiccation tolerance in somatic embryos (111). In microspore-derived embryos of *Brassica oleracea* (broccoli), desiccation tolerance was induced by the application of ABA during maturation, with the highest tolerance depending on the age and size of the embryos and the concentration of exogenous ABA (128).

As with dormancy, the role of ABA in desiccation tolerance is still debatable. Although, the application of ABA clearly induces desiccation tolerance in seed tissues, the mechanisms involved are still a mystery.

Reserve Mobilization

During seed development, reserve proteins are laid down in the embryo and megagametophyte (endosperm) (1), and it is not until germination that these proteins are mobilized by the synthesis of appropriate degradative enzymes such as α-amylase. ABA (and osmotica) have been implicated in the control of these mobilization enzymes. Alpha-amylase is a GA-induced enzyme that is retarded at the transcription level by the addition of ABA to culture. Removal of ABA by washing also induces the production of α-amylase. But, ABA may not be the only critical factor in reserve mobilization. In maize, fluridone treatments

only slightly increased α-amylase production and drying was necessary to "sensitize" the kernels to GA. Therefore, it appears that ABA as an inhibitor to reserve mobilization is only part of the puzzle (46,48).

Physiological Effects of ABA in Conifer Culture

A comparison of the cellular and biochemical patterns of zygotic and somatic embryogenesis in conifers, and the effects of ABA in conifer culture, was recently reviewed by Attree and Fowke (10), as well as, by Tautorus et al. (1), and the recent status of somatic embryogenesis in conifer forestry has been reviewed by Becwar (56). Overall, the majority of ABA's actions in conifer systems appear to be similar to those discussed in the above sections for non-coniferous species. A summary of critical studies follows.

Although ABA is not a requirement for every *in-vitro* conifer system (45,56), incubation of conifer somatic calli in the presence of ABA prior to transferring to a hormone-free media typically results in increased density of immature embryos (precotyledonary) and improved embryo development and maturation (cotyledonary) (56) with somatic embryos following an identical developmental pathway as their zygotic counterparts (1,10,129). It has been suggested that ABA suppresses cleavage polyembryony in *Picea sitchensu* (sitka spruce), *Pseudotsuga menziesii* (Douglas fir), and *Picea abies* (Norway spruce), thereby permitting further development of individual somatic embryos (1,10). ABA also appears to inhibit precocious germination in *Picea glauca-engelmanni* (interior spruce) (1,130), *Picea glauca* (white spruce) (131), and Douglas fir (57). Incubation of interior spruce (129,130) and white spruce (1,132) somatic embryos in ABA resulted in the accumulation of lipids, proteins,

carbohydrates, and in the case of Norway spruce and *Picea glauca* white spruce, triacylglycerides (1,56,132). The patterns of accumulation and degradation of storage products in interior spruce (1,129,130) and Norway spruce during development and germination of somatic embryos were identical to those found in the corresponding zygotic forms suggesting similar storage product types, functions, biosynthesis, and metabolism (1). Addition of ABA has also been found to improve desiccation tolerance in Norway spruce (57) and white spruce embryos (131,132). In loblolly pine, the addition of ABA to a low osmolarity maintenance media appeared to improve early embryo quality when organized structures were already present (133).

As is typical in most tissue culture systems, extended experimentation performed in a "trial-and-error" manner has been the hallmark for conifers. The optimal concentration, timing of the exposure, and the length of exposure to ABA varied depending on species, genotype within species, developmental stage of somatic embryos at the time of ABA exposure, presence of other PGHs, such as GAs (134) or ratios of auxin/cytokinins during initiation (10), and possibly the endogenous level of ABA in the somatic embryos (135). Effective ABA concentrations in maturation media have been reported from 0.025 to 60 μM (1,10,134), although higher levels are becoming more common as it appears that the cultures lose sensitivity to ABA with time leading to precocious germination (10). ABA is generally added to suppress proliferation and to induce differentiation and development of embryos. Although several protocols are used to induce development in conifer embryos, a common element is the reduction or elimination of auxin either by charcoal addition or transfer to an auxin-free media prior to ABA addition (136,137,138,139,140). After exposure to ABA, calli

are typically transferred to hormone-free media which induces further embryo development (56).

Other culture conditions which have been found to facilitate conifer embryo development and maturation include addition of activated charcoal (presumably to remove waste products and to slowly reduce the ABA concentrations) during ABA exposure for Douglas fir and loblolly pine, and Norway spruce (56,57,141); use of ABA in combination with IBA (56), cytokinin (142), or gibberellin (134) for Norway spruce and Douglas-fir; and partial drying at high humidity after ABA treatment for interior spruce (1). The application of low molecular weight (MW) plasmolyzing osmotica, such as sucrose, glucose, or salt to Douglas fir, loblolly pine, and Norway spruce (143) or high MW non-plasmolyzing osmotica, such as polyethylene glycol (PEG) to white spruce (131) have all been found to have profound affects on somatic embryo development (10). The combination of high MW osmotica + ABA has been shown to be superior to ABA or osmotica alone in suppressing precocious germination, enhancing storage product accumulation, and inducing desiccation tolerance (57,131,132,144). ABA did not appear to act by indirectly mediating the water relations (as has been suggested for many angiosperms), as osmotic stress alone did not substitute for ABA (144). This supports the theory that ABA acts on gene expression directly (10) and not by controlling water stress. Incorporation of 5% PEG-4000 and ABA in white spruce somatic maturation media increased maturation frequency 3-fold, increased the size and distribution of storage protein and lipid bodies, and induced desiccation tolerance (10). For loblolly pine, somatic embryos incubated on maturation media containing 5 ppm ABA and 13% PEG showed faster growth rates, improved embryo development, and increased the

number of cotyledonary embryos in comparison to media supplemented only with ABA (145). Research indicated that storage protein synthesis in somatic conifer embryos is stimulated by ABA, but because only the combination of PEG + ABA resulted in the full complement of storage proteins, osmoticum must also regulate the protein synthesis in this system (10). High MW PEG + ABA has similar affects on triacylglycerol (TAG) synthesis in white spruce (1).

CONCLUSIONS

From the foregoing review of the literature, it should be evident that ABA influences a wide array of physiological, biochemical, and genetic events during seed and embryo development. These events include promotion of embryo morphogenesis, storage protein synthesis, and desiccation tolerance, as well as control of the onset and maintenance of dormancy. Inhibition of precocious germination and storage protein mobilization are also events that are regulated by ABA. In many angiosperm and gymnosperm culture systems, the application of exogenous ABA is required for the continued normal development of somatic embryos.

Most of these putative roles for ABA have been suggested using causal relationships between endogenous ABA levels and some event, or by applied exogenous ABA and, therefore, must be viewed with caution. However, the use of ABA mutants, which have allowed for the manipulation of endogenous ABA levels and sensitivities, have supported ABA roles in the above events. In the future, as ABA-regulated genes are further identified and isolated, the role of ABA, and how it operates on a molecular level, will be understood.

Almost all our understanding of how ABA functions in conifer systems have been through the addition of exogenous ABA to somatic cultures. Fortunately, the majority of ABA's actions in the conifer system appear to be similar to those in non-coniferous species. However, in order to encourage the production of mature conifer somatic embryos, a more direct approach would be more expedient and cost-effective. It is suggested that the growth conditions (media composition) should approximate the natural *in-ovulo* conditions throughout all developmental stages from induction to germination. An improved understanding of induction is desirable so that important conifer species, e.g., loblolly pine, that have remained unresponsive to *in-vitro* development and maturation, can be encouraged.

PROBLEM ANALYSIS AND OBJECTIVES

Very little is known about the changes in ABA levels, or for that matter, many of the other biochemical and molecular events that occur during conifer zygotic embryogenesis. In fact, there is no published literature pertaining to changes in ABA during zygotic development in any gymnosperm. However, from what evidence exists, ABA appears to play a critical role in the proper development of zygotic and somatic conifer embryos.

THESIS OBJECTIVES

The objective of the research presented herein was to measure the changes in ABA concentration in the seed tissues of loblolly pine during zygotic embryogenesis and to better understand the role played by ABA in determining the major morphological events. This was accomplished by:

1) developing techniques to collect, extract, and store seed tissues from loblolly pine cones. These techniques involved separation of embryo, megagametophyte, and suspensor tissues and determining their proper developmental stage prior to long-term storage,
2) developing techniques to extract, purify and analyze ABA concentration of the individual seed tissues. These techniques included development of a highly sensitive routine indirect enzyme-linked immunosorbant assay (ELISA) for ABA and methods for validating the ELISA estimates, and
3) measuring the ABA concentrations in embryo, megagametophyte, and suspensor tissues during zygotic embryogenesis in loblolly pine during 1992

and 1993, as well as determining if causal relationships exist between ABA levels and morphological and physiological events during loblolly pine seed development.

As PGHs likely play critical roles in zygotic embryogenesis phases, *stricto sensu* embryogenesis, growth phase, and maturation phase, it is possible to assume that valuable comparisons could be made between ABA levels determined during natural and somatic embryogenesis. Therefore, results from this study have potential use in developing a model to assist in determining the optimal ABA requirements for a somatic embryogenesis system, as well as those studies requiring seed formation and ripening information on conifers.

The initial research proposal also included plans to analyze IAA during zygotic embryogenesis in loblolly pine seed tissues. As the research progressed, this goal became increasingly impractical and was thus removed as an objective. Nevertheless, the results for extraction, purification, and quantitation using GC/MS are presented in Appendix 3.

CHAPTER TWO - DEVELOPMENT OF AN INDIRECT ELISA FOR QUANTITATING ABA IN LOBLOLLY PINE DEVELOPING ZYGOTIC TISSUE

INTRODUCTION

Loblolly pine zygotic tissues are very small in size and weight, and most likely contain picogram concentrations of ABA. Thus, a routine analytical technique with a high degree of sensitivity to ABA was required. The major goal of this research was, therefore, to develop a quantitative method capable of accurate estimates of endogenous ABA in the zygotic tissues of loblolly pine. To complete this goal, several procedures were developed, including methods to extract, purify, and store zygotic tissues until analysis could be performed. An amplified indirect enzyme-linked immunosorbant assay (ELISA) for ABA and techniques to validate the ABA estimates, such as GC/MS-SIM, were also required.

MATERIALS AND METHODS

EXTRACTION AND PURIFICATION MATERIALS AND METHODS

Plant Materials

Open pollinated loblolly pine cones (two to six) from genetically superior mother trees were obtained from seed orchards from Westvaco Corporation, Summerville, SC (one mother tree; WV_{91} [genotype 240]) and Union Camp Corporation, Bellville, GA (one mother tree; UC_{91} [genotype 10-68])) during 1991. Cones were shipped overnight, on ice packs, on a weekly basis from early June to late October. Methods for dissection, storage, and staging of

zygotic tissues are detailed in Chapter Three under Plant Tissue Methods.

Extraction and Purification Materials

Methanol was HPLC grade from J.T. Baker Chemical (Phillipsburg, NJ) and water was either deionized or distilled. Antioxidants, butylated hydroxytoluene (BHT) (B-1378) and sodium diethyldithiocarbamate (DDC) (D-3506), were obtained from Sigma (St. Louis, MO) as was Tween 20 nonionic detergent (P-1379). The extraction solvent was 80% methanol (v/v) in deionized water containing 25 mg/L BHT which had been adjusted to pH 7.0-7.5 using diluted HCl. Tritiated (50 μCi) abscisic acid (DL-*cis,trans*-[G-^{3}H]-ABA) (TRK.644) and aqueous bio-scintillation cocktail were obtained from Amersham (Arlington Heights, IL). Forty mL screw cap-type Teflon extraction tubes were obtained from Nalgene (Rochester, NY). Prepacked C_{18} reverse-phase preparatory columns and 15 mL solvent reservoirs were obtained from J.T. Baker Chemical. Vacuum loading of preparatory C_{18} columns was accomplished using a Baker-10 SPE™ vacuum manifold equipped with luer fittings (J.T. Baker Chemical) capable of utilizing an aspirator or pump as a vacuum source. Filtering discs (Acrodisc No. 4438) with luer tips and fabricated with a nylon filtering membrane (0.45 μm pore size) were obtained from Fisher Scientific (Pittsburgh, PA). Roto-evaporation was accomplished using a VV-Micro micro/semi-micro rotary evaporator (Wheaton, Millville, NJ). Scintillation counting was performed using a LS3801 Scintillation Counter (Beckman, Irvine, CA) programmed to convert counts per minute (CPM) to disintegrations per minute (DPM) using a set of ^{3}H quench samples (Beckman). All counting was performed to ±0.5% standard deviation.

Extraction and Purification Methods

Methods development included determination of the type and degree of purification required for ELISA and GC/MS analysis using radioactive isotopes and successive approximation, and initial validation of the ELISA using additivity and parallelism. Final validation was by GC/MS. Methods for determining false positives using preparatory C_{18} cartridges, and HPLC techniques for additional purification of ABA are also described.

ABA Extraction Methods

Lyophilized tissue was removed from the freezer (-80°C) and quickly ground in the glass storage ampoule using a cold (4°C) glass rod. Tissues representing identical stages of development from a single mother tree were combined (bulked) into a single ampoule prior to grinding to facilitate homogenization. Bulking increased the available tissue per stage; this was especially important for early staged tissues, which had very little mass. The ground tissue was quickly weighed on a microbalance, then transferred to a Teflon extraction tube containing 10 mL of chilled (4°C) extraction solvent plus an internal standard of approximately $5x10^5$ DPM of [^{3}H]-ABA and a stir bar. For determination of ABA recovery, 100 µL of this solvent, prior to the addition of tissue, was pipetted into glass scintillation vials containing 15 mL scintillation cocktail and counted. This represented 100% recovery of the initial DPM (see Equation 1). The tubes were purged with pre-purified nitrogen gas to reduce the likelihood of oxidation, capped, and vortexed to wash any tissue clinging to the sides of the tubes into the extraction solvent. The tissues were extracted in the dark overnight with constant stirring at 4°C.

ABA Purification Methods

The following purification techniques were considered: filtration through nylon, preparatory reverse-phase C_{18} column chromatography, and reverse-phase C_{18} HPLC. Two purification solvent protocols were considered: 1) acidic, and 2) neutral aqueous methanol. Tritiated ABA was counted to determine which of the purification protocols would result in the highest percentage recovery. After selection, successive approximation was used to determine the extent of purification required for the ELISA and GC/MS. Purification required for ELISA was validated by additivity and parallelism (tests to detect interferences in the immunoassay), as well as by direct comparison to GC/MS. A detailed method is described below for each protocol.

Purification Method 1 - Acidic Aqueous Methanol. Ten mL chilled extraction solvent (4°C) plus approximately 1000 DPM ^{3}H-ABA (no plant tissue) was reduced to an aqueous phase by roto-evaporation *in-vacuo* at 30-35°C under minimal lighting. The aqueous phase was transferred to a plastic filtration tube connected to the appropriate purification scheme: nylon filter only or nylon filter + preparatory C_{18} column, each set on the vacuum manifold (Fig. 8).

The roto-evaporation flask was rinsed three times with 2 mL aliquots of chilled 1% acetic acid + 100 mg/L BHT (4°C), and the rinses were subsequently transferred to the plastic filtration tube. The preparatory C_{18} column was washed and activated prior to use by addition of two 5-mL aliquots of chilled 100% methanol followed by two 5-mL aliquots of chilled 1% acetic acid. All purification trials were performed at 4°C (cold room) under minimal lighting.

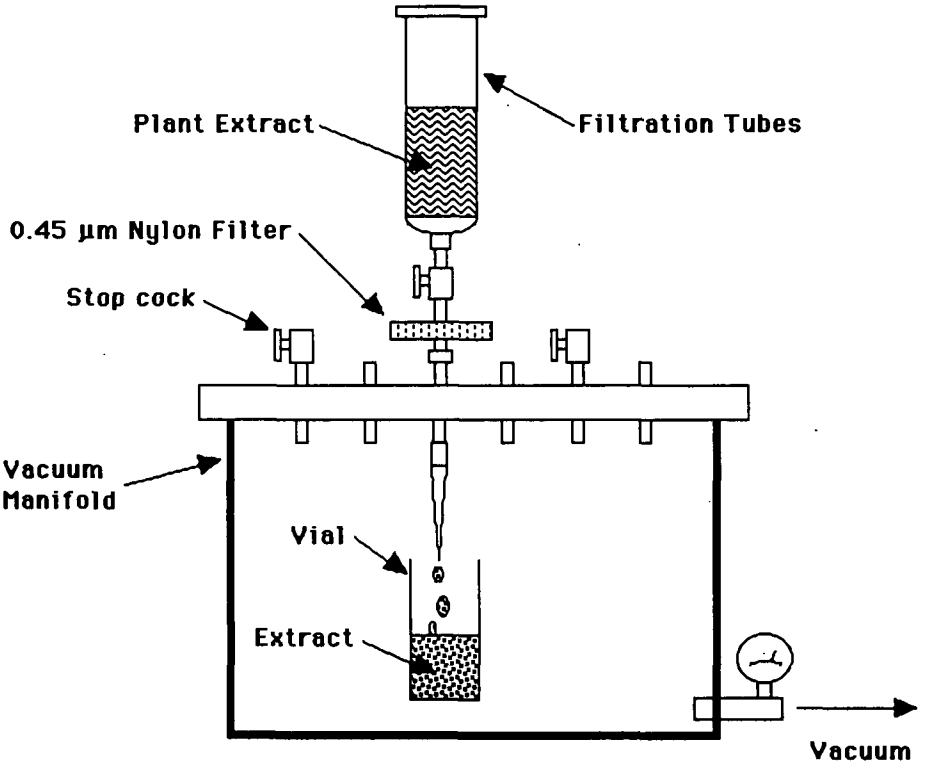

Figure 8. Vacuum manifold fitted with nylon filter and C_{18} chromatography column.

Vacuum (<5 in. Hg) was applied to the manifold and the extracts were allowed to collect into scintillation vials at a rate of 1 mL/minute or less. Two trials were performed to maximize ABA recovery through the C_{18} column. In Trial No. 1, the following scheme was used: the first pass was collected, a wash of 4 mL chilled 0.1 M acetic acid (4°C) was collected, and the fraction containing ABA was eluted from the column using 6 mL chilled 40% methanol in 0.1 M acetic acid. In Trial No. 2, the following scheme was used: the first pass was collected, a wash of 5 mL chilled water (4°C) was collected, and the fraction containing ABA was eluted from the column using 10 mL 100% chilled methanol (4°C). Portions (100 μL) from each collection were removed for scintillation counting and percent recovery was determined using Equation 1.

$$\% \ Recovery = \frac{(Volume\ of\ Extract_{Final})\ x\ (DPM_{Final})}{(Volume\ of\ Extract_{Initial})\ x\ (DPM_{Initial})}\ x\ 100\%$$

Equation 1. Percentage recovery determination from scintillation counting.

Purification Method 2 - Neutral Aqueous Methanol. Three trials were performed to measure the ABA recovery using a neutral extract. Ten mL of chilled extraction solvent (4°C) plus approximately 1,000 DPM ^{3}H-ABA and no plant tissue (Trials No. 3 and 4) was reduced to an aqueous phase by roto-evaporation *in-vacuo* at 30-35°C under minimal lighting. A sample containing ground Stage 9 whole ovules (approximately 0.5 g dry-weight), after overnight extraction and centrifuging at 2000 x *g* for ten minutes, was treated identically (Trial No. 5). The extracts were transferred to plastic filtration tubes connected to the appropriate purification scheme; nylon filter alone or nylon filter + preparatory C_{18} column, each set on the vacuum manifold. The extraction tubes were rinsed with three aliquots of chilled water (pH adjusted to 7.0-7.5) to obtain a final methanol concentration of 70% (v/v) and the washes were transferred to the appropriate plastic filtration tube. The preparatory C_{18} column was washed and activated prior to use by addition of two 5-mL aliquots of chilled water followed by two 6-mL aliquots of chilled 70% methanol + 100 mg/L BHT. Vacuum was applied to the manifold (<5 in. Hg) and the extracts were collected into scintillation vials at a rate of 1 mL/minute or less. In the case of the preparatory C_{18} cartridge, Trial Nos. 3-5 used the following scheme: the first pass was collected and two washes with 2.5 mL chilled 70% methanol (v/v) + 100 mg/L BHT were each collected separately. Portions (100 µL) were removed from each collected pool for scintillation counting and percent recovery

determination.

HPLC Methods

All HPLC methods were performed on a liquid chromatograph (Model 5000, Varian, Sugarland, TX) equipped with a single wavelength UV monitor (UV_{254}) connected to a Hewlett Packard model 3390A integrator (Wilmington, DE). Separations were performed on a reverse-phase Econosphere C_{18} (250 mm x 4.6 mm i.d. x 5 μm) HPLC column (Alltech Assoc., Deerfield, IL). For ABA purifications, as well as determination of methylation efficiencies for GC/MS analysis, samples were dissolved in 500 μL of 30% (v/v) methanol adjusted to pH 3.0 using acetic acid, of which 250 μL was injected onto the column. The mobile phase was a linear gradient from 30% (v/v) methanol (pH 3.0) to 100% methanol over a 20 minute interval at a flow rate of 0.9 mL/minute. Retention times (R_t) for ABA and for methyl-ABA were approximately 15.9 and 18.7 minutes as determined using authentic ABA and methylated ABA.

For determining the efficiency of ABA methylation by diazomethane, authentic ABA was methylated as described in the GC/MS-SIM Materials and Methods Section and injected onto the HPLC column as described above. Appearance of a peak at a R_t corresponding to ABA was taken to indicate incomplete methylation. HPLC analysis was also used to determine whether ABA was degraded during the extraction and purification methods. For this, a known amount of ^{3}H-ABA diluted in solvent and injected onto the HPLC column. The sample was eluted using an isocratic solvent system of 30% acetonitrile/70% RO water, adjusted to pH 3.0, and the eluent was collected as 0.5 mL fractions in scintillation vials

containing 15 mL scintillation cocktail. Radioactivity in the recovered fractions was subsequently counted. For comparison, the same amount of ^{3}H-ABA was run through the extraction and purification method (nylon filter + preparatory reverse-phase C_{18} column) detailed above, and labeled ABA in the effluent was counted.

Assessing the Degree of Purification Required for GC/MS Quantitation of ABA

Determination of the purification requirements for GC/MS-SIM analysis of ABA was performed using two methods. First, plant extracts (Stage 9 ovules) spiked with ABA were left unpurified, were purified by nylon filter, or by nylon filter + preparatory reverse-phase C_{18} column prior to GC/MS-SCAN and SIM analysis. The appropriate clean-up techniques were judged from the complexity of the resultant chromatograms. Once the appropriate purification protocol was selected, a further check of sample cleanliness was performed by monitoring the protonated molecular ion of methyl-ABA at m/z 190 together with the major fragment ion at m/z 162 during GC/MS-SIM. Peak area ratios (PAR) of 190/162 for pure ABA were compared to those for sample ABA, and similar ratios were taken to indicate adequately purified samples (146).

Successive Approximation for Determination of Purification Requirements for ELISA

The following purification techniques were considered: no purification, nylon filtering, nylon filtering + preparatory reverse-phase C_{18} column, and nylon filtering + preparatory reverse-phase C_{18} column + reverse-phase C_{18} HPLC. After optimal purification solvent protocol (i.e., acidic or neutral solvent) was selected, successive approximation was

used to determine the extent of purification required for the ELISA. Ten (10) Stage 9 ovules were ground and extracted following the extraction method, except that 30 mL of chilled (4°C) extraction solvent were used. After extraction, the samples were centrifuged at 2000 x *g* for ten minutes, and the supernatant was divided into three 10-mL portions. Each portion was further divided into nine 1-mL portions for replication of each purification setup, i.e., three 1-mL aliquots were tested in each of the purification procedures (crude extract, nylon filter only, and nylon filter + preparatory C_{18} column).

ABA INDIRECT ELISA MATERIALS AND METHODS DEVELOPMENT

ABA Indirect ELISA Materials

The monoclonal antibody (mAb) raised to free *cis*-(+)-ABA as described by Mertens et al. (147), was obtained from Idetek, Inc. (San Bruno, CA). The ABA-C'_4-bovine serum albumin conjugate (ABA-BSA) was prepared according to Quarrie and Galfre (148). Goat anti-[mouse antibody]-biotin conjugate (GAM-biotin) (B-7264), tetramethyl benzidine (TMB) substrate (T-2885), bovine serum albumin (BSA) (A-3425), and *cis,trans*-(±)ABA (A-1012) were procured from Sigma. Streptavidin-Poly-20HRP conjugate (strep-HRP) (#RDI-pHRP20-SA) was acquired from Research Diagnostics, Inc. (Flanders, NJ). All other chemicals used in the making of buffers were purchased in the purest available form and were used without additional purification. Water was deionized or reverse osmosis (RO) grade. Buffers and reagents were made as follows (34,149):

PBS + Tween (phosphate-buffered saline + Tween 20): 13.6 g (100 mM) potassium monophosphate (K_2HPO_4) and 29.2 g (500 mM) sodium chloride (NaCl) per liter of water

containing 500 µL Tween 20 [0.05% (v/v)] were adjusted to pH 7.4 using potassium hydroxide (KOH), stored at room temperature (RT), and made fresh every other week.

Blocking buffer: 10 g BSA in 200 mL PBS + Tween 20 [5% (w/v)], was stored at 4°C and made fresh every week.

Assay buffer: 20 mL blocking buffer diluted into 980 mL PBS + Tween 20 [0.1% (v/v)], was stored at 4°C and made every week.

Sodium carbonate buffer: 4.2 g (0.05 M) sodium carbonate (Na_2CO_3) per liter of water, adjusted to pH 9.6 using sodium hydroxide (NaOH), was stored at RT and made fresh every other week.

Substrate buffer: 8.2 g (500 mM) sodium acetate ($NaC_2H_3O_2$) per liter of water, adjusted to pH 5.5 using dilute citric acid, was stored at RT and made fresh every other week.

Enzyme substrate: 520 µL of TMB stock solution (10 mg/mL of TMB in dimethyl sulfoxide [DMSO]) and 29 µL of 3% hydrogen peroxide (H_2O_2) were diluted into 52 mL of substrate buffer and used immediately.

Monoclonal antibody: Anti-ABA mAb (2 mg) was dissolved in 2 mL assay buffer (1 mg/mL final concentration), aliquoted into 10 µL portions (10,000 ng) and stored at -80°C. The mAb was observed to be stable for at least one year under these conditions. Prior to use in the ELISA, the mAb was further diluted in assay buffer to a final concentration previously determined by checkerboard titration trials.

ABA-C'$_4$-BSA conjugate (ABA-BSA): ABA-BSA was synthesized by Dr. Jeffrey Dean (University of Georgia, Athens, GA) and had a final antigen density of approximately 1.4 x 10^{13} ABA molecules/well on a microtiter plate (protein not determined). This was calculated

by first determining the amount of ABA that would bind all the anti-ABA mAb in the assay, i.e., effective B_{max}, by extrapolating the standard curve to the abscissa or log [ABA]. This was approximately 3.2 ng/100 µL ABA. Assuming that all of the ABA molecules in the ABA-BSA conjugate bind to the anti-ABA mAb, there would be (200 µL x 3.2 ng/100 µL) of ABA/well in the microtiter plate. The conjugate was prepared in a two part synthesis and is briefly prepared as follows: *ABA-C'$_4$-ABH-HCL Synthesis:* 4-aminobenzoyl hydrazide (ABH; 15.5 mg) was dissolved in 3.05 mL of 1 M methyl formate (MeCOOH) in methanol and vortexed until dissolved. (±)-ABA (13.5 mg) was added to the solution, which was then vortexed, purged with nitrogen gas, and incubated at RT for three days in the dark. The MeOH was removed under nitrogen resulting in a yellow oil which was partitioned twice against 0.1 M borate buffer (BB; boric acid solution adjusted to pH 9.0) and ethyl acetate (AcOEt). The AcOEt washes were discarded and the aqueous phase was adjusted to pH 4.5, followed by several partitions against AcOEt. The AcOEt extracts were combined and taken to dryness under nitrogen gas (yellow residue). The residue was resuspended in 200 µL MeOH and 800 µL 0.1 M HCl (clear solution). *ABA-C'$_4$-ABH-BSA Synthesis:* The following solutions were prepared and cooled to 0°C on ice: BSA, 160 mg/8 mL 0.1 M BB dialyzed overnight against 0.25 M BB, adjusted to pH 9.2; $NaNO_2$, 75 mg/3 mL water; ABA-C'$_4$-ABH-HCl, 500 µL. The following steps were performed on ice. $NaNO_2$ (70 µL) was added drop wise to the ABA-C'$_4$-ABH-HCl and diazotization was allowed to proceed for 20 minutes (yellow color). The resultant solution was added drop wise to 1.5 mL of the BSA solution and incubated overnight at 2°C under darkness (orange-brown color). This solution was dialyzed against 0.1 M BB, adjusted to pH 9.0 for three days at 4°C under darkness. The

remaining ABA-C'$_4$-ABH-HCl was added to the ABA-C'$_4$-ABH-BSA with $NaNO_2$ (70 µL) and diazotization proceeded for a further 20 minutes. This solution was added to 1.5 mL of the BSA solution, incubated overnight at 2°C under darkness, and then was dialyzed for two days at 4°C under darkness. The resultant ABA-C'$_4$-ABH-BSA (abbreviated ABA-BSA) conjugate was aliquoted at full strength into 10 µL portions for storage at -20°C. The conjugate was observed to be stable for at least three years under these conditions. Prior to use in the ELISA, the conjugate was further diluted in sodium carbonate buffer to an optimal concentration (1:100,000), as determined by checkerboard titration trials.

Goat anti-[mouse antibody]-biotin conjugate (GAM-biotin): Aliquoted into 10 µL portions and stored at -20°C. The conjugate was diluted 1:5,000, four times the dilution recommended by the manufacturer, in assay buffer (37°C) prior to use in the ELISA.

Streptavidin-Poly-20HRP (strep-HRP): Aliquoted into 10 µL portions and stored at -20°C. The conjugate was diluted 1:5,000, as determined by checkerboard titration trials, in assay buffer (37°C) prior to use in the ELISA.

ABA standards: A stock solution of 5,000-6,000 ng/µL *cis*-(+)-ABA (higher concentrations caused ABA to precipitate visibly as a white powder on the lip of the container) was prepared by dissolving *cis,trans*-(±)-ABA in absolute methanol and assuming that equal amounts of (+)-ABA and (-)-ABA were present. This solution was observed to be stable for at least three months when stored at -20°C in a screw-capped brown glass vial, wrapped in Parafilm, and covered with aluminum foil. Standards ranging from 15.8 ng to 5 pg/100 µL (+)-ABA were made in assay buffer by serial exponential dilution. The assay range was 2.5 ng to 2.5 pg/100 µL for (+)-ABA.

Extraction solvent: 80% methanol (v/v) in deionized water, containing 25 mg/L BHT as an antioxidant, and adjusted to pH 7.0-7.5 using diluted HCl, was allowed to sit 24 hours after make-up to allow BHT to fully dissolve. This solvent was stored at RT and made fresh every month.

Equipment: Immulon 2, flat-bottom, 96-well microtiter plates (Dynatech Laboratories, Chantilly, VA) were utilized for optimal binding of the ABA-C'_4-BSA conjugate (54). The outer rows and columns have been shown to produce inconsistent results (150,151), so only the inner 60 wells on each plate were used. Parafilm (American National Can, Greenwich, CT) was used to cover the microtiter plates during all incubations.

All washing steps were performed using an EL401 manual microplate washer (Bio-tek, Instruments, Inc. Winooski, VT). Enzyme reaction product absorbances were measured at 650 nm and 450 nm using a EL312E Bio-Kinetics microtiter plate reader (Bio-tek) interfaced to an AT-class 286 IBM-compatible computer. The plate reader was controlled by Kineticalc II software (Bio-tek), and data was analyzed using Quattro Pro, v. 3.0 software (Borland International). Statistical analysis was performed using NCSS (Kaysville, UT) or SAS (SAS Institute, Cary, NC).

ABA Indirect ELISA Methods

A flow chart for the indirect ELISA procedure is shown in Fig. 9. The protocol was basically modified from Maldiney et al. (55) by use of streptavidin-poly-20HRP conjugate to increase the sensitivity of the assay towards ABA. Details of the procedure follow.

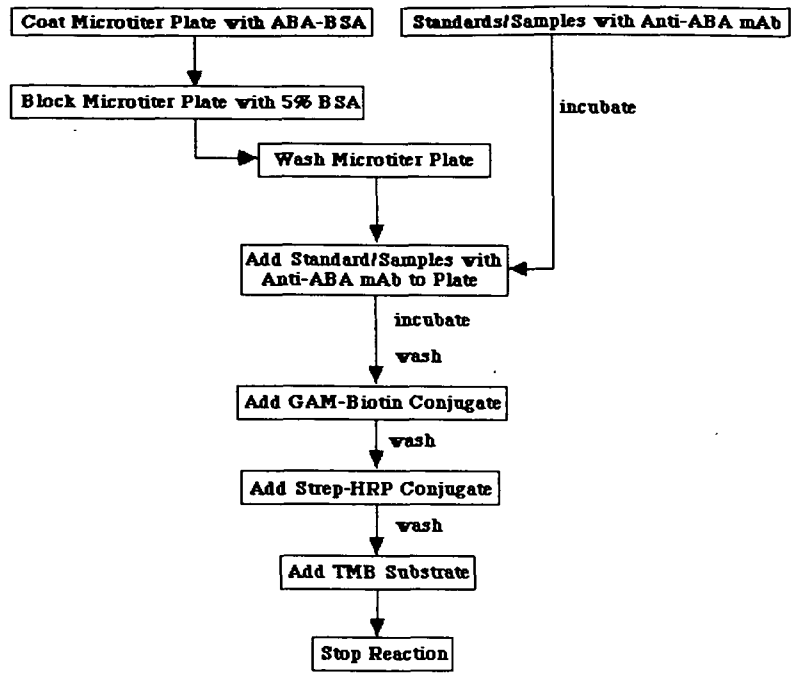

Figure 9. Flow chart for the ABA indirect ELISA.

Coating of the microtiter plate: Diluted ABA-BSA conjugate (200 µL) was added to each well of the microtiter plate, except those in the outside rows and columns as they were previously shown to produce inconsistent results (150,151). The plates were sealed with Parafilm and incubated overnight at 4°C in the dark.

Blocking of the wells: The wells were aspirated and washed four times with PBS + Tween. Approximately 300 µL of blocking buffer was added to each well, the plates were resealed in Parafilm, and left to incubated for at least 45 minutes at 37°C in the dark. The wells were aspirated and washed four times with PBS +Tween.

Incubation of standards or samples with ABA antibody: ABA standards and samples were combined 1:1 with the diluted mAb and incubated overnight at 4°C in the dark. This was performed concurrently with coating of the microtiter plates. Samples were diluted in

assay buffer to obtain absorbances in the center of the calibration curve (≈50% binding). Aliquots (200 µL) from the samples or standards incubated with monoclonal antibody were added to the plate, which was then sealed in Parafilm, and incubated for 90 minutes at 37°C in the dark. The wells were aspirated and washed four times with PBS + Tween.

Addition of GAM-biotin: Diluted GAM-biotin (200 µL) was added to each well, the plate was sealed in Parafilm, and incubated for 90 minutes at 37°C in the dark. The wells were aspirated and washed four times with PBS + Tween.

Addition of Strep-HRP: Diluted strep-HRP (200 µL) was added to each well, the plate was sealed in Parafilm, and incubated for 90 minutes at 37°C in the dark. The wells were aspirated, washed four times with PBS + Tween, and once with 300 µL substrate buffer to remove chloride ions (inhibitory to peroxidase activity).

TMB reaction: Enzyme substrate (200 µL) was added to each well, and the blue color was allowed to develop at RT in the dark until a B_o absorbance of 0.35-0.45 at 630 nm was detected using the Bio-Tek microplate reader. Reactions were stopped with 40 µL of 1.5 N H_2SO_4 and the absorbances at 450 nm were immediately recorded.

Optimization of the ABA Indirect ELISA

The ELISA was optimized to assure that the incubation conditions were saturated for, but not wasteful of, expensive chemical components, to maximize sensitivity of the assay, to decrease assay time, and to reduce variability of the results. Optimized incubation time and temperature minimized variability due to changes in RT conditions while maintaining sensitivity and reducing assay time.

ELISA Reagents. Checkerboard titrations were used to determine the optimal dilutions (i.e., saturating conditions) for the ABA-BSA conjugate and anti-ABA mAb. Checkerboard titrations were performed by running a decreasing dilution series of mAb along the columns of a microtiter plate and a decreasing dilution series of ABA-BSA along the rows as shown in Fig. 10. All other reagents were used as described in the Indirect ELISA Materials Section.

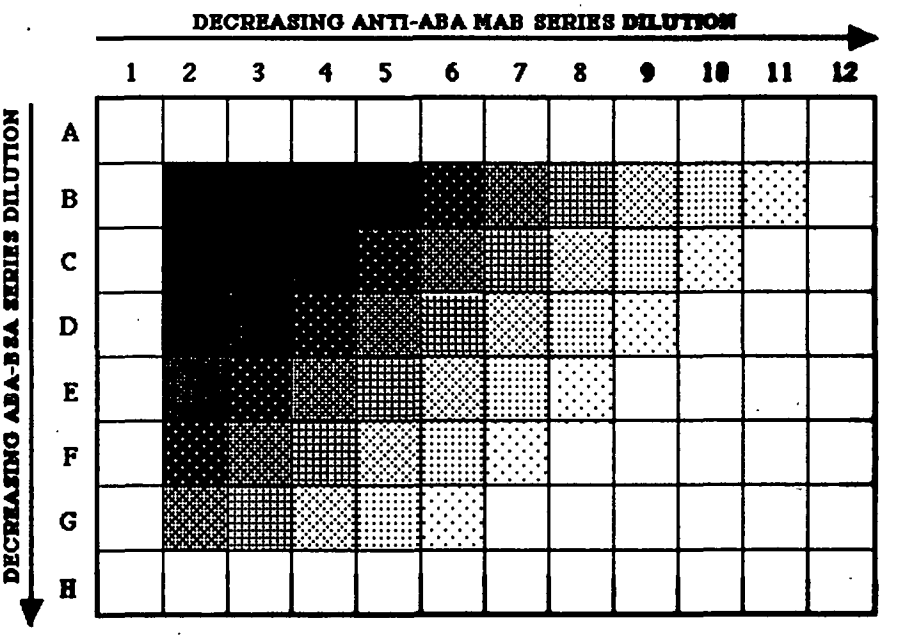

Figure 10. Idealized checkerboard titration for optimizing ABA-BSA and anti-ABA mAb concentrations. Intensity of grey-scale represents absorbance intensity of colored reaction products.

Optimum dilutions for ABA-BSA and anti-ABA mAb were obtained under the following criteria: 1) final absorbance of 1.3-1.5 O.D. as measured at 450 nm after addition of H_2SO_4; 2) the least amount of mAb required to reach this absorbance; and 3) the lowest ABA-BSA concentration at the optimal mAb concentration which did not significantly alter the final absorbance ("saturated").

After optimal concentrations were determined for ABA-BSA and anti-ABA mAb, a series dilution of strep-HRP was performed to determine its optimal dilution. The GAM-biotin was not optimized as it was very inexpensive and would likely expire before its depletion.

Incubation Temperature and Time. During the initial development of the ELISA, incubations were RT and two hours, for the anti-ABA mAb, secondary antibody conjugates, and the strep-HRP. For blocking with 5% BSA, temperature and time were RT and one hour, respectively. The TMB reaction was at 37°C for 20-30 minutes (until final absorption was obtained).

After optimization of the reagents, incubation temperature was increased to 37°C (17,36) while incubation times were decreased to 90 minutes for mAb, secondary antibody conjugates, strep-HRP, and 45 minutes for blocking. The following optimized conditions shown in Table 2 were adopted for the indirect ELISA during tissue analysis.

Sensitivity of the ABA Indirect ELISA

Under the optimized conditions adopted for the ELISA, ABA standards were made by serial exponential dilution from 800 to 0.0005 ng/100 μL (final concentrations of 400 to 0.00025 after addition of mAb solution) and fit to a linear model using the least squares method (see below). The linear range used for quantitation was determined using the correlation coefficient (r^2) after confirming random residual errors. ELISA sensitivity was determined as the absorbance that was less than minus (-) 95% confidence interval of B_o (17).

Table 2. Incubation temperature and time optimization experiments.

ELISA Reaction	Initial Experiments		Optimizing Experiments	
	Temperature, °C	Time, min.	Temperature, °C	Time, min.
Blocking	RT	60	37	30
Anti-ABA mAb + Standard/Sample	RT	120	37	90
GAM-Biotin	RT	120	37	90
Strep-HRP	RT	120	37	90
TMB	37	variable	RT	variable

Data Analysis

In this section, procedures on generating a standard curve and its linearization by logit transformation will be discussed. Calculation of the amount of ABA present in unknown samples will also be discussed.

Construction of the Standard Curve. The standard curve was first constructed by calculating the percentage binding (B_i/B_o, %) for each ABA standard using Equation 2 and can be can be plotted against the natural log (Ln) of ABA concentration to produce a curvilinear function as shown in Fig. 11. To improve the linear range of the standard curve, the ABA standards were transformed using the logit transformation shown in Equation 3. One should be aware that linearization using this technique will generally lead to an artificial compression of errors at either end of the curve (36). The logit (B_i/B_o) for each standard is plotted against the natural log (ln) of its concentration as shown in Fig. 12 (38,39). A least square fit linear

$$B_i/B_o,\ \% = \frac{(B_i - B_o)}{(B_{max} - B_o)}\ x\ 100\%$$

where: B_i = Absorbance of each standard (and sample).

B_o = Absorbance at 100% binding of antibody (0 ng/100 µL ABA).

B_{max} = Absorbance at 0% binding of antibody (7.9 ng/100 µL ABA).

Equation 2. Determination of percentage binding in the ABA indirect ELISA.

$$Logit(B_i/B_o) = Ln[\frac{(B_i/B_o,\ \%)}{(100 - B_i/B_o,\ \%)}]$$

Equation 3. Logit transformation equation for linearizing the standard curve.

regression model is fit to the data and this model is used to determine the ABA concentration (ng/100 µL) in the samples using sample absorbances after logit transformation.

Determination of Sample ABA. After the plant extract was purified and taken to near dryness *in vacuo* at 30-35°C under minimal lighting, the remaining aqueous phase was diluted to exactly 1000 µL in assay buffer. Exactly 100 µL of this extract was pipetted into a glass scintillation vial containing 15 mL scintillation cocktail for determination of ABA recovery. Serial dilutions of the remaining extract (~900 µL) were made in assay buffer to prepare at least two of the following samples (total volume was dependent on the number of replications of each dilution desired and concentration of ABA in sample): full strength (x1), half

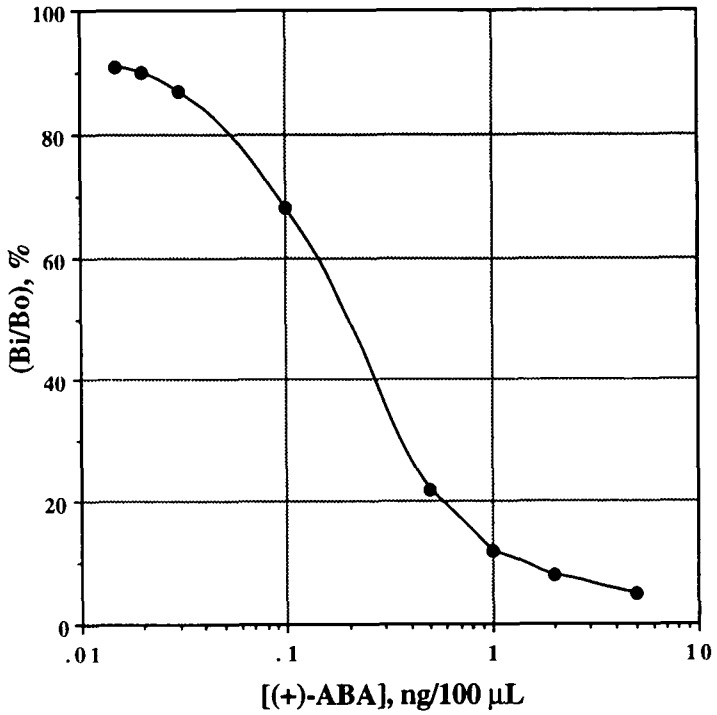

Figure 11. Example of a non-linearized standard curve for the ABA indirect ELISA.

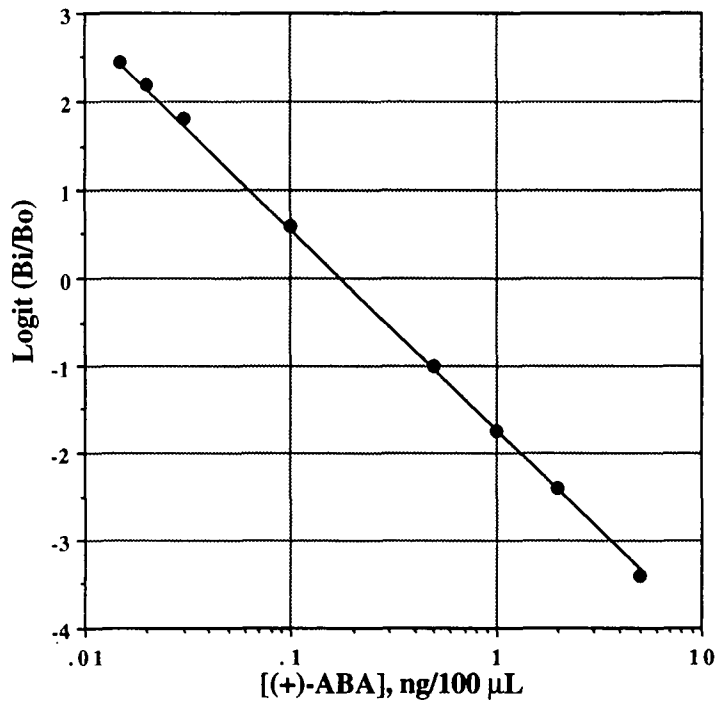

Figure 12. Example of a transformed linearized standard curve for the ABA indirect ELISA.

strength (x2), and quarter strength (x4), e.g., 400 µL extract (x1), 200 µL extract + 200 µL assay buffer (x2), and 100 µL extract + 300 µL assay buffer (x4), respectively, or any dilution within this range, i.e., x1 to x4. Dilutions were made to obtain final sample absorbances near the middle of the standard curve ($B_i/B_o \approx 50\%$ binding). Diluted anti-ABA mAb was added to each sample in equal volume (1:1), e.g., in the example above, 400 µL extract to 400 µL antibody, and allowed to incubate overnight as outlined in the ELISA protocol. Final ABA estimates were corrected for losses during purification as determined from loss of radioactive ^{3}H-ABA using the following equation:

$$\% \ Recovery = \frac{(1 \ mL \ x \ DPM_{final})}{(10 \ mL \ x \ DPM_{initial})} \ x \ 100\%$$

where 1 mL represents the sample volume after purification and roto-evaporation and 10 mL represents the volume of extraction solvent.

In the final purification scheme, a blank, i.e., extraction solvent + ^{3}H-ABA only, was also purified and treated as a sample extract. This blank was used to correct for the addition of ^{3}H-ABA, as well as to indicate any problems in the assay results, e.g., high absorbance in blank.

The concentration of (+)-ABA (ng/100 µL) in the sample extracts was calculated by performing the percent binding (B_i/B_o) and logit transformation on each sample absorbance and comparing the results to the standard curve (included in every plate) using the linear model that best fit the curve. The concentration of (+)-ABA in the plant tissue (ng/g dry-

weight) was estimated by taking into account the extract dilutions during the ELISA and purification, as well as the amount of tissue extracted, and correcting for the blank and losses during purification.

Precision and Accuracy of the ABA Indirect ELISA

Precision in Purification and ABA Indirect ELISA Methods

Variability of the ELISA quantitation protocol was estimated in two steps: 1) ELISA alone, and 2) purification + ELISA. Variability in the ELISA alone (Trial Nos. 1 and 2) was first measured by assaying an ABA standard at a dilution (B_i/B_o)≈ 50% binding of the antibody (approximately 0.25 ng ABA/100 μL extraction solvent). This provided an estimate of the assay variability in the absence of interfering agents from the plant tissue.

Trial 3 was performed with plant tissue and the procedure is illustrated in Fig. 13. A single sample (0.5 g dry-weight) of Stage 9 (9LL) ovules (see Chapter Three) was extracted and purified (nylon filter) as a single sample as outlined in the Extraction and Purification Methods Section. After purification, the extract was subdivided into seven equal aliquots, which were dried simultaneously under a stream of pre-purified nitrogen and minimal light at RT. Each aliquot was then assayed in triplicate for ABA at (B_i/B_o)≈ 50% binding. These trials estimated the within-run precision of the ELISA. Variability in the combined purification and ELISA procedure was also determined using Stage 9 (9LL) ovules. Variability of the combined purification and ELISA methods was determined as illustrated in Fig. 14, and was tested on two separate days (Trial Nos. 4 and 5).

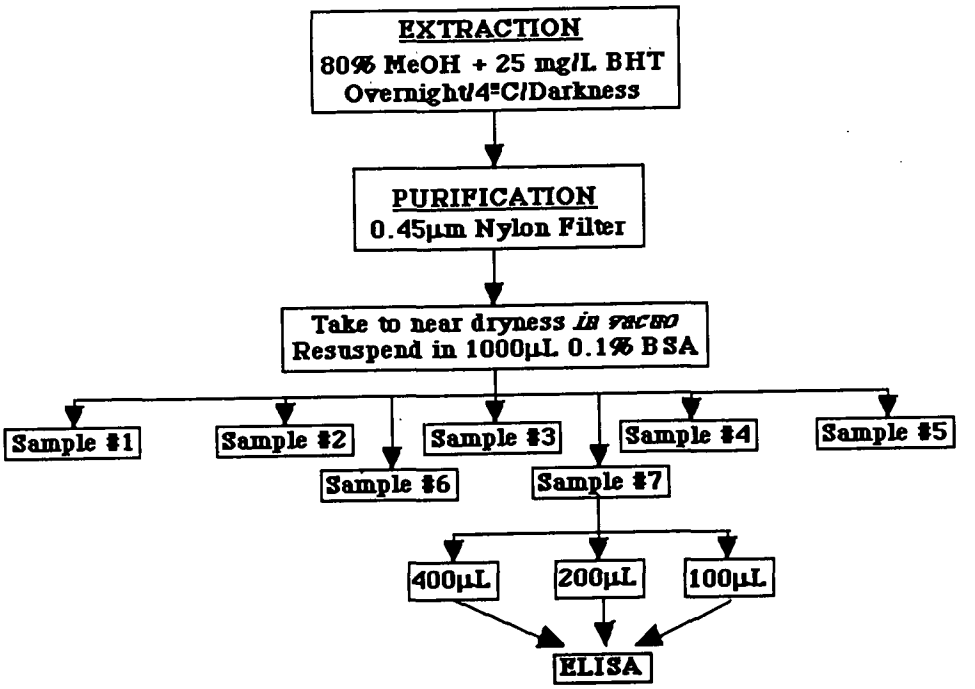

Figure 13. Protocol for determining variability in the ABA indirect ELISA alone.

A single tissue sample (0.5 g dry-weight) was extracted as outlined in the Extraction Methods Section, subdivided into seven equal aliquots, and each aliquot was purified individually (nylon filter). All aliquots were simultaneously dried under pre-purified nitrogen gas and minimal lighting at RT. Each aliquot was assayed for ABA (in triplicate) to $B_i/B_o \approx$ 50% binding of the antibody. Trial Nos. 3, 4, and 5 were used to estimate the between-run precision for the entire quantitation method.

To account for plate-to-plate variability within and between runs, a standard ABA curve was included in every plate. B_o and B_{max} were determined using at least four replicates and ABA standards were run in triplicate.

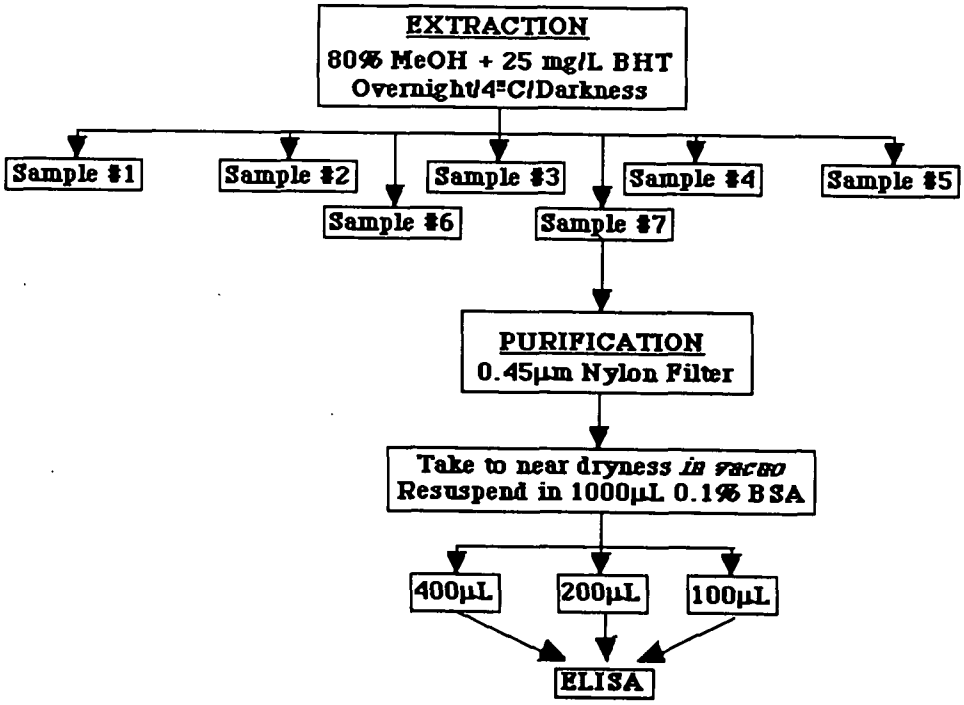

Figure 14. Protocol for determining variability in purification and the ABA indirect ELISA.

Accuracy and Checks for Interference of the ABA Indirect ELISA

The most influential sources of interference in immunoassays are: (1) presence of strongly cross-reacting compounds similar to the antigen, i.e., ABA; (2) excessive amounts of weakly cross-reacting compounds similar to the antigens; (3) presence of antibody denaturing or desorbing materials, i.e., solvents; (4) presence of factors which prevent the antibody-antigen binding, e.g., detergents; and (5) presence of interferences which limit the quantitation (enzyme assay) steps (27). Table 3 lists the relative cross-reactivity of a variety of synthetic compounds and phytochemicals similar in structure to ABA (39). As the table illustrates, the monoclonal antibody is quite specific towards the 2-*cis*-(+)-ABA molecule.

Table 3. Cross-reacting compounds to the ABA mAb (38).

Compound	% Cross-reacting
2-*cis*-(+)-ABA	100.00
2-*cis*-(+)-ABA methyl ester	<0.10
2-*cis*-(-)-ABA	0.00
2-*trans*-(+)-ABA	0.00
2-*cis*-(+)-ABA-B-D-glucopyranosyl ester	0.00
Phaseic acid	<0.10
Dihydrophaseic acid	<0.10
Xanthoxin	0.00
All-trans-Farnesol	0

There are two main approaches employed to ascertain the accuracy of immunoassay-based quantitative estimates for PGHs: tests for additivity and for parallelism (28). These approaches can indicate gross interference types, such as those that might be found in Nos. 2-5 above (27).

Additivity. The test of additivity is a simple technique to detect interference in the immunoassay. If additivity is present, i.e., twice the quantity of extract assayed produces twice the value of ABA, then interference is not present. Stated differently, the final ABA estimate, after correcting for dilution, should result in the same value. A dilution series of purified tissue extracts from Stage 3, 8B, or 9 megagametophytes were made in assay buffer at the following dilutions: x1, x2, and x4 (i.e., extract diluted 1:1, 1:2, and 1:4 in buffer) and assayed for (+)-ABA using the indirect ELISA.

Parallelism. The test of parallelism to the standard curve was performed by assaying a constant amount of tissue extract in the presence of increasing amounts of exogenous ABA. If there are no interfering substances in the plant extracts, a line parallel to the standard curve with a y-intercept corresponding to the amount of endogenous ABA present in the extract is obtained (28). To purified extracts of early and late stage megagametophytes, various amounts of exogenous (+)-ABA (0, 25, 50, 100, 200, 400, and 600 pg/μL) was added at two dilutions of extract: 1:12.5 and 1:25 for the early (bulked Stage 4 & 5), and 1:25 and 1:50 for the late (bulked Stage 9) megagametophytes. The seven samples were then assayed using the indirect ELISA, and the resulting curves depicting (+)-ABA added versus (+)-ABA estimated by the indirect ELISA were compared to a standard curve generated with the same exogenous ABA.

GC/MS-SIM MATERIALS AND METHODS DEVELOPMENT

GC/MS-SIM Materials

Deuterated abscisic acid, $^2H_6$-ABA (roughly 1.8 μg) (synthesized by Dr. Laurent Rivier) was a gift from Dr. Richard Pharis, University of Calgary, and was stored dry at -20°C under a nitrogen atmosphere in the dark. Hexane was GC-grade solvent (J.T. Baker Chemicals) and helium carrier gas was high-purity grade (Holox, Atlanta, GA). All other solvents were of the highest purity and were used without additional purification. Water was deionized or RO grade. Vials (100 μL) with screw caps and Teflon septa were obtained from Fisher Scientific.

Analysis of ABA was performed on a Hewlett-Packard 5890 Series II Gas Chromatograph (GC) with direct capillary interface to a Hewlett-Packard 5971A mass-selective (quadrapole) detector (MSD) (Wilmington, DE). The capillary column was a 30 m x 0.25 mm x 0.25 μm film DB-1 (J&W Scientific, Ltd.). GC/MS was integrated to a 386/25 IBM-compatible computer controlled by Microsoft Chemstation (Microsoft, Redmond, WA).

GC/MS Methods

Synthesis of Diazomethane

Methylation of ABA standards and samples was performed using diazomethane generated from diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide, CH_2N_2). Preparation of 1-100 mM ethereal diazomethane in alcohol from diazald was performed in the fume hood using the Diazald® kit as described by Aldrich (152,153). Diazald and diazomethane are carcinogenic compounds and solutions of diazomethane may explode violently upon heating, bright light, or on rough glass surfaces. The above references should be reviewed before diazomethane synthesis so that all safety precautions are implemented. The only modification to the diazald method was that a receiving flask and ether trap were not used. Ether from the condensation tube was allowed to drip into a Erlenmeyer flask containing glacial acetic acid. Diazomethane, identified by its deep yellow color, was synthesized in 50-60 mL batches. These were collected during synthesis into aluminum foil covered scintillation vials, where diazomethane appeared stable for up to one month when capped scintillation vials were wrapped in Parafilm and kept in the dark at -20°C. Methylating efficiency was determined by HPLC as described earlier under HPLC Methods.

Methylation of Standards and Samples

The chemical reaction for methylating ABA is illustrated in Fig. 15. Standards and samples in 100 µL vials with screw caps and Teflon septa were taken to dryness under a gentle stream of pre-purified nitrogen gas using hand warming and minimal lighting. Large

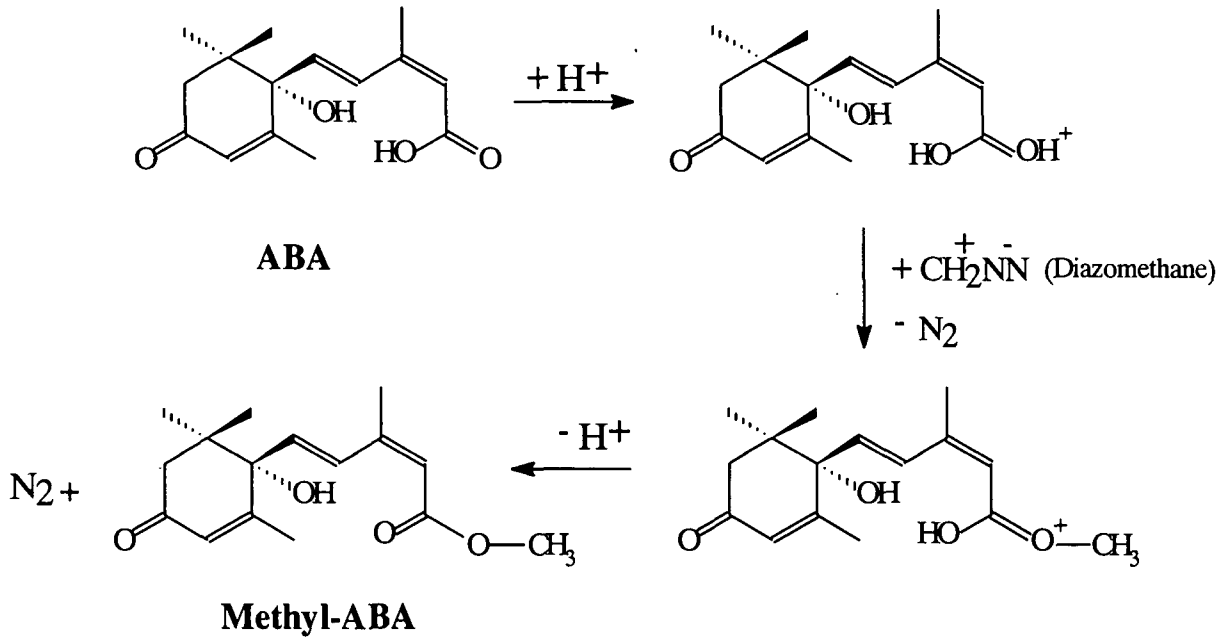

Figure 15. Methylation of ABA using diazomethane.

samples (>100 µL) were taken to dryness by roto-evaporation *in-vacuo* at 30-35°C, resuspended in <100 µL of methanol, transferred to 100 µL vials, and then dried under pre-purified a nitrogen gas stream. In a ventilated hood, ethereal diazomethane was added to the vials using Pasteur pipets with fire-polished tips. The vials were then capped and gently shaken. Caps were then loosened to allow nitrogen gas to escape, and the vial was placed in a ventilated hood in the dark. If the yellow color of the diazomethane persisted after 20 minutes, the

sample was assumed to be fully methylated and was subsequently reduced to dryness under a pre-purified nitrogen gas stream using hand warming and minimal lighting. If after 20 minutes the solution was no longer yellow, the sample was reduced to dryness under a pre-purified nitrogen gas stream and dim lighting, and additional diazomethane was added. This procedure was continued until the yellow color persisted longer than 20 minutes. The dried samples were stored at -20°C, under a pre-purified nitrogen atmosphere, and in the dark until ready for dilution in hexane prior to GC/MS analysis (≤ two days).

GC/MS Method of Analysis

Standards and purified samples of ABA used for validating the indirect ELISA were quantified by GC/MS-SIM using enriched stable isotope $^{2}H_{6}$-ABA as an internal standard (Fig. 16) (13,14,109). The analysis protocol method modified from Dunlap and Guinn (31) for optimal quantitation of ABA on the GC/MS instrument at the IPST. Sample introduction (1 μL) was made through a splitless injector with and interface temperature of 300°C. The GC temperature program was 60°C for two minutes, followed by a 25°C/minute increase to 165°C, then a 5°C/minute increase to 275°C. The helium gas carrier flow rate was 0.5 mL/minute with a column head pressure of 8 psi. MS conditions were set for an ionization potential of approximately 70 eV with an ion source temperature of 300°C.

Full-SCAN mode was used to identify ABA and to detect any contaminants in the plant samples. Quantitation data were collected using the SIM program. Retention times (R_t) for ABA and $^{2}H_{6}$-ABA were approximately 12.22 and 12.19 minutes, respectively.

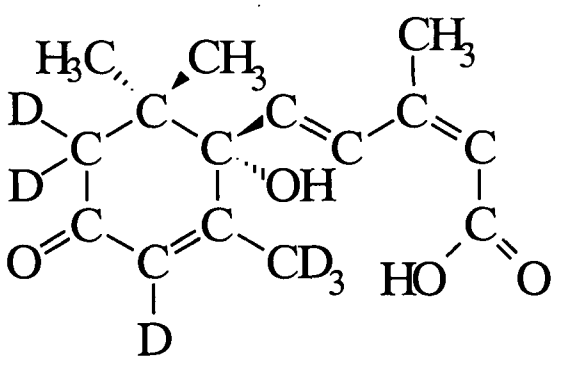

Figure 16. Chemical structure of enriched stable isotope $^{2}H_{6}$-ABA used as an internal standard in GC/MS quantitation of ABA. D denotes deuterium atoms (^{2}H) replacing H atoms.

Development of the GC/MS Standard Curve Using Stable Isotope Dilution Technique

ABA was analyzed by monitoring peak areas of the two major fragment ions of Me-ABA: m/z 190 and 162 for comparison with the corresponding ions from Me-$^{2}H_{6}$-ABA (m/z 194 and 166, respectively). Figure 17 illustrates the electron impact mass spectrum for methyl-ABA showing the two major fragmentation ions at m/z 190 and 162. Peak areas ratios of m/z 190 to 194, measured at the retention times of the corresponding reference substances, were used in all quantitation calculations. Peak areas ratios of m/z 190 to 162 were used to monitor impurities in sample extracts by comparing to peak area ratios for a methylated ABA standard. On each day that samples were to be analyzed, a calibration plot was constructed from ABA standards having concentration ratios (CR) of ABA to $^{2}H_{6}$-ABA ranging from 5:1 to 1:8. The peak area ratios (PAR) between the m/z 190 and 194 molecular ions (ABA and $^{2}H_{6}$-ABA, respectively) in these standards covered the ABA concentration ranges anticipated in the unknown samples. Calibration standards were made in 100 µL

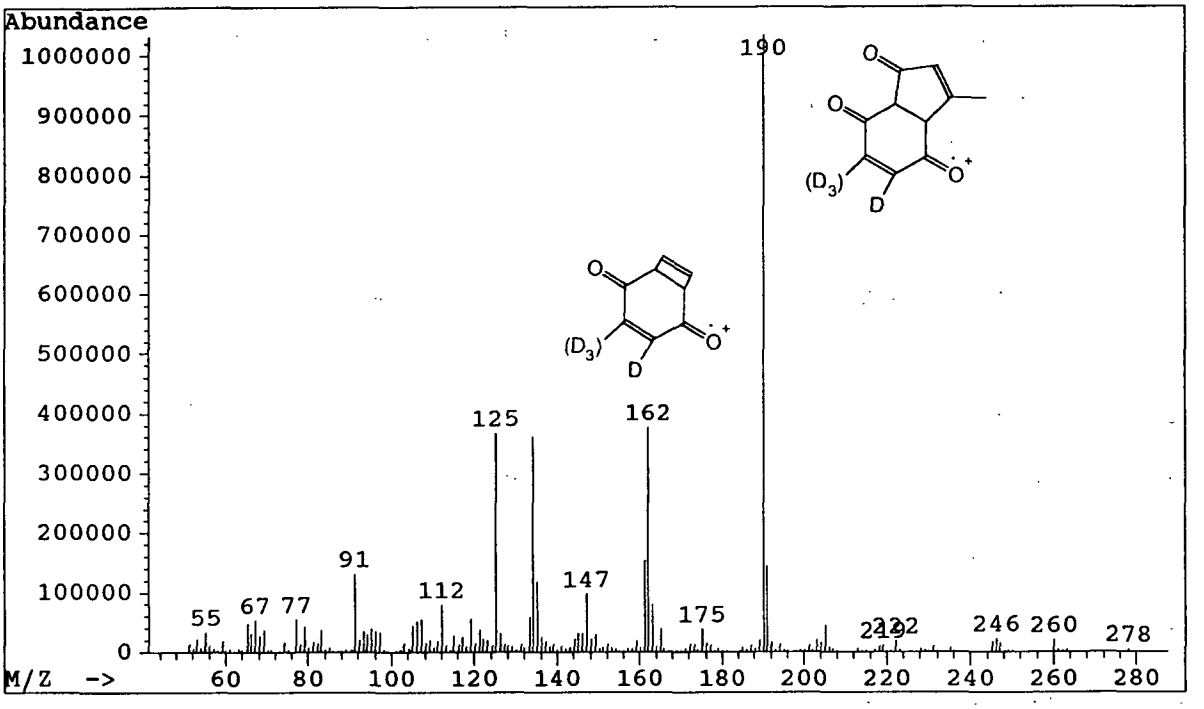

Figure 17. GC/MS-SCAN fragmentation spectra for methyl-ABA from 55 to 300 m/z. Structures shown refer to fragments at m/z 162 and 190. (D) and (D_3) denote possible locations for deuterium in the $^2H_6$-ABA (m/z 166 and 194).

microvials as follows: 1) 50 µL of ABA standard (1000, 800, 600, 400, 200, 100, 50, and 25 pg/µL) was added to each vial; 2) 10 µL of $^2H_6$-ABA (approximate concentration of 1000 pg/µL) was added; 3) samples were dried under pre-purified nitrogen gas with hand warming and minimal lighting; 4) samples were fully methylated using diazomethane; 5) dried under pre-purified nitrogen gas with hand warming and minimal lighting; and 6) diluted in 50 µL hexane. These samples contained ABA concentrations as listed above with an estimated $^2H_6$-ABA content of approximately 200 pg/µL.

The areas of the molecular ion peaks for ABA (m/z 190) and $^2H_6$-ABA (m/z 194) were integrated, and the mean ratios of 190/194 for each standard combination were calculated. Each combination was prepared and methylated once for each standard curve and

subsequently injected two or three times on the GC/MS each day of analysis. Recovery rates between the ELISA and GC/MS procedures were assumed to be identical as radioactive isotopes were not allowed on the GC/MS.

Determination of the GC/MS Sensitivity and Range of Linearity

Sensitivity and the range of linearity of the GC/MS-SIM were determined through construction of the calibration curve. The ability of the GC/MS to integrate areas of peaks having heights three to ten times higher than background (154) was used to estimate the sensitivity. The range of linearity was confirmed using the correlation coefficient (r^2) of the least square linear regression curve.

Determination of Enriched Stable Isotope $^2H_6$-ABA Concentration

The concentration of the $^2H_6$-ABA was determined during the construction of the calibration plot. It was assumed that when the integrated peak areas of (±)-ABA and $^2H_6$-ABA were equivalent, i.e., 1:1, then so were their concentrations.

Determination of Endogenous ABA Concentrations in Zygotic Tissues

Prior to methylating samples, $^2H_6$-ABA was added to the purified plant extracts at a concentration approximately equal to the amount of endogenous ABA as estimated following the procedure outlined for standards in the Development of GC/MS Standard Curve Using Stable Isotope Dilution Techniques Section. Endogenous ABA was analyzed by monitoring the protonated molecular ion of Me-ABA (m/z 190) together with the major fragment ion

(m/z 162) as well as the corresponding ions from Me-$^2H_6$-ABA (m/z 194 and 166, respectively). PAR of m/z 190 to 194, measured at the retention times of the corresponding reference substances, were used in all quantitative calculations. In theory, the PAR of m/z 190 to 194 multiplied by the amount of $^2H_6$-ABA added would equal the amount of endogenous ABA originally present in the sample extract. In practice, however, a standard curve relating PAR of m/z 190/194 to CR of m/z 190/194 must be constructed. This is because the isotopic composition of the deuterated ABA is not 100% labeled, i.e., there may be unlabeled ABA present in the $^2H_6$-ABA. Thus, the $^2H_6$-ABA standard will contribute ions at m/z 190 (13) which required correction for accurate quantitation. PAR of m/z 190 to 162 were used to monitor impurities in sample extracts by comparison to PAR for a methylated ABA standard. The concentrations of endogenous ABA in the extracts were determined using Equation 4 (14,109,146). Determination of endogenous ABA in the plant tissues was accomplished by back calculating using dilution factors and the amount of plant tissue extracted.

$$(\pm)ABA,\ pg/\mu L = (Conc.\ Ratio\ of\ m/z\ 190/194) \times internal\ std.\ added,\ pg/\mu L$$

Equation 4. Determination of endogenous ABA using enriched stable isotope dilution.

Validation of the ABA Indirect ELISA via GC/MS

Individual samples of plant tissue from Stages 4, 8B, and 9 (see Chapter Three) were extracted, purified, and taken to dryness by roto-evaporation *in vacuo* at 30-35°C under

minimal lighting. The residue was resuspended in 1000 µL absolute methanol and divided into two 500 µL portions, one for ELISA and the other for GC/MS. The ELISA sample was dried with a stream of pre-purified nitrogen gas with hand warming and minimal lighting. The sample was resuspended in 1000 µL assay buffer, and analyzed for ABA as detailed above. Figure 18 illustrates the procedure to validate the ELISA using GC/MS. Three tissues were validated so as to cover a range of tissues at various stages of development: early (Stage 4), middle (Stage 8B) 13 replications, and late (Stage 9) stage tissues (21 replications).

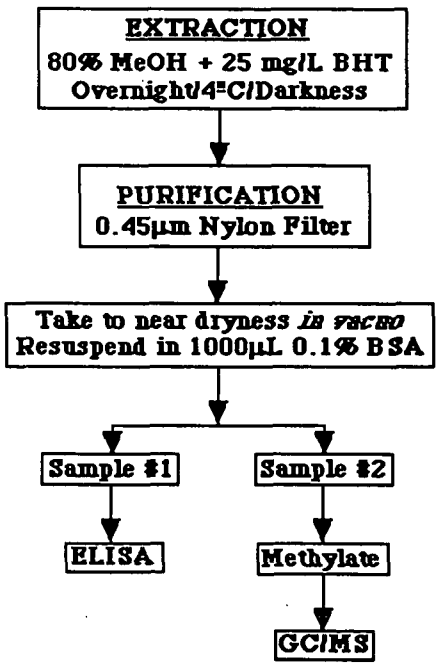

Figure 18. Protocol for validation of the ABA indirect ELISA using GC/MS.

The sample for GC/MS analysis was dried with a stream of pre-purified nitrogen gas with hand warming and minimal lighting. A quantity of enriched stable isotope ($^2H_6$-ABA in methanol) equivalent to the endogenous ABA level, as determined by ELISA, was added to the sample. The combined sample was fully methylated using ethereal diazomethane

and dried under a stream of pre-purified nitrogen gas with hand warming and minimal lighting. Samples were diluted in hexane so that the ABA and $^{2}H_{6}$-ABA concentrations would fall in the middle of the calibration curve (approximately 500 pg/μL). Six replicates were made for the early- and mid-developmental stages and ten replicates for the late developmental stages. As samples containing radioactive isotopes could not be analyzed on the GC/MS, recovery was considered to be identical to that determined for the ELISA.

STATISTICAL ANALYSIS

Statistical analysis of successive approximation, effects of dilution ratios on ELISA estimates, and validation of the ELISA was based on Schuirmanns' equivalence method for two-sided t-tests (155). This method is considered to be more powerful than the student's two-sided t test for detecting equivalent means. ELISA sensitivity was determined using the Student's two-sided t-test. Analyses were typically carried out at 95% confidence intervals ($\alpha = 0.05$).

RESULTS

EXTRACTION AND PURIFICATION

Selection of Extraction Method

The extraction method was based on techniques used most often in the current literature (11,13,14). The optimal extraction solvent was found to be 80% methanol in deionized or reverse osmosis (RO) water containing 25 mg/L BHT and adjusted to pH 7.0-7.5 (neutral methanolic solution) (13,16,23). Tissues were extracted at a volume to weight ratio of 500:1 to 2000:1 (mL extraction solvent:g dry-weight).

Selection of Purification Method

Table 4 displays the percent recovery of ^{3}H-ABA determined for the acidic and neutral purification solvent trials. Trial Nos. 1 and 2, using acidic solvent without plant tissue, resulted in variable and usually low recovery rates. Trial Nos. 3 and 4 resulted in excellent recovery of the tritiated ABA in the absence of plant tissue. Trial No. 5, to which plant tissue was added for the extraction steps, showed slightly less recovery than Trials Nos. 3 and 4. This was expected given the potential for trapping of ABA in the plant tissues. The neutral solvent system was selected for purification because of the uniformly high recovery rates obtained. The lack of a significant difference in recovery of ^{3}H-ABA in Trial Nos. 3-5 also suggested that nylon filtering alone might be sufficient for purifying pine embryo extracts.

Table 4. Selection of a purification solvent system.

Solvent Type	Trial No.	Plant Tissue?	Purification	% Recovery[a] (± Std. Dev.)
Acidic	1	No	Nylon filter	69 ± 4
			Nylon filter + Prep. C_{18} column	58 ± 3
	2	No	Nylon filter	91 ± 5
			Nylon filter + Prep. C_{18} column	53 ± 3
Neutral	3	No	Nylon filter	100 ± 5
			Nylon filter + Prep. C_{18} column	95 ± 5
	4	No	Nylon filter	100 ± 5
			Nylon filter + Prep. C_{18} column	98 ± 5
	5	Yes	Nylon filter	94 ± 5
			Nylon filter + Prep. C_{18} column	87 ± 4

a: Based on scintillation counting of tritiated (^3H) ABA.

Degradation of ABA during Extraction and Purification

As shown in Fig. 19, HPLC fractionation was used to determine the degree of ABA degradation during extraction and clean-up. The assumption was that if the radioactive peaks had identical retention times without the appearance of new peaks, it could be concluded that there was insignificant degradation of ABA during the extraction and purification protocols. As shown, the before and after peaks have identical retention times with no additional peaks following extraction and purification. Thus, there appeared to be no significant degradation of ABA during these procedures.

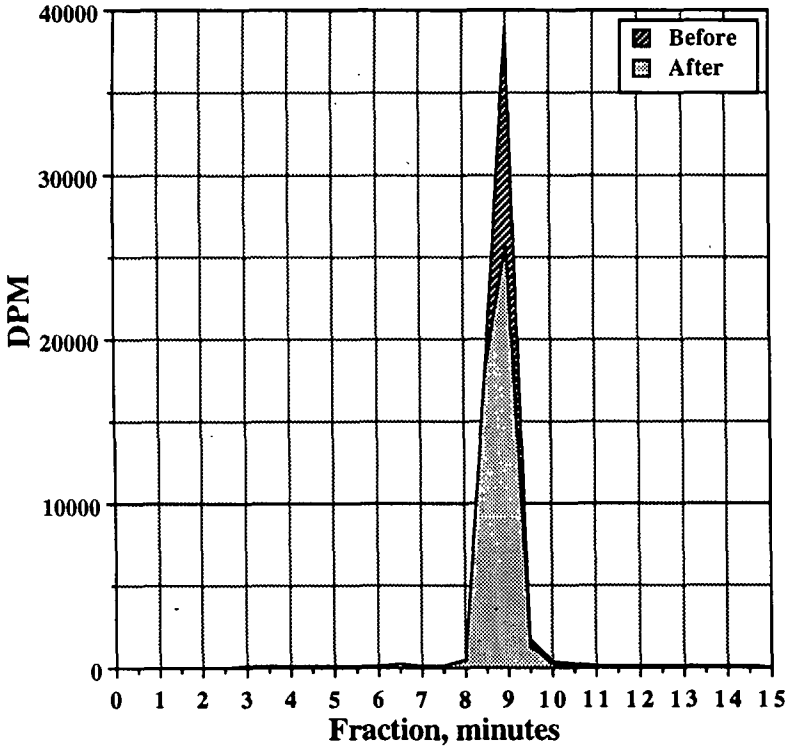

Figure 19. Fractionation of ^{3}H-ABA via HPLC before and after extraction and purification procedures. Difference in peak areas is due to loss of ^{3}H-ABA during clean-up (81% recovery).

Ascertaining the Degree of Purification Required for GC/MS Quantitation of ABA

ABA-spiked (1 ng/μL (+)-ABA) plant extracts were analyzed using GC/MS-SCAN (Fig. 20) and GC/MS-SIM (Fig. 21) after the following purification steps: A) none; B) nylon filtering; and C) nylon filtering + preparatory C_{18} column. Nylon filtering alone removed the majority of the background contamination inherent to the unfractionated extract. The addition of a preparatory C_{18} column step did not appear to improve the purity of the extract with respect to GC/MS analysis. Therefore, nylon filtering alone was considered adequate purification for quantitating pine zygotic ABA by GC/MS. Quantitative successive approximation was not performed for GC/MS.

As a second check, the PAR of m/z 190 to 162 was calculated for each sample and standard subjected to GC/MS-SIM. Table 5 compares the PAR of m/z 190 to 162 between pure ABA, and ABA in extracts from Stages 4, 8B, and 9 megagametopytes. As the table illustrates, there was no statistical difference in purity between the filtered plant extracts and the ABA standards. Mean values for Stages 4 and 8B extracts were much closer to the ABA standards than to Stage 9 extracts, presumably because Stage 9 tissue extracts contained higher levels of contaminants than did extracts from Stages 4 and 8B. Consequently, nylon filtering was considered adequate purification for GC/MS quantitation of ABA from loblolly pine zygotic tissue.

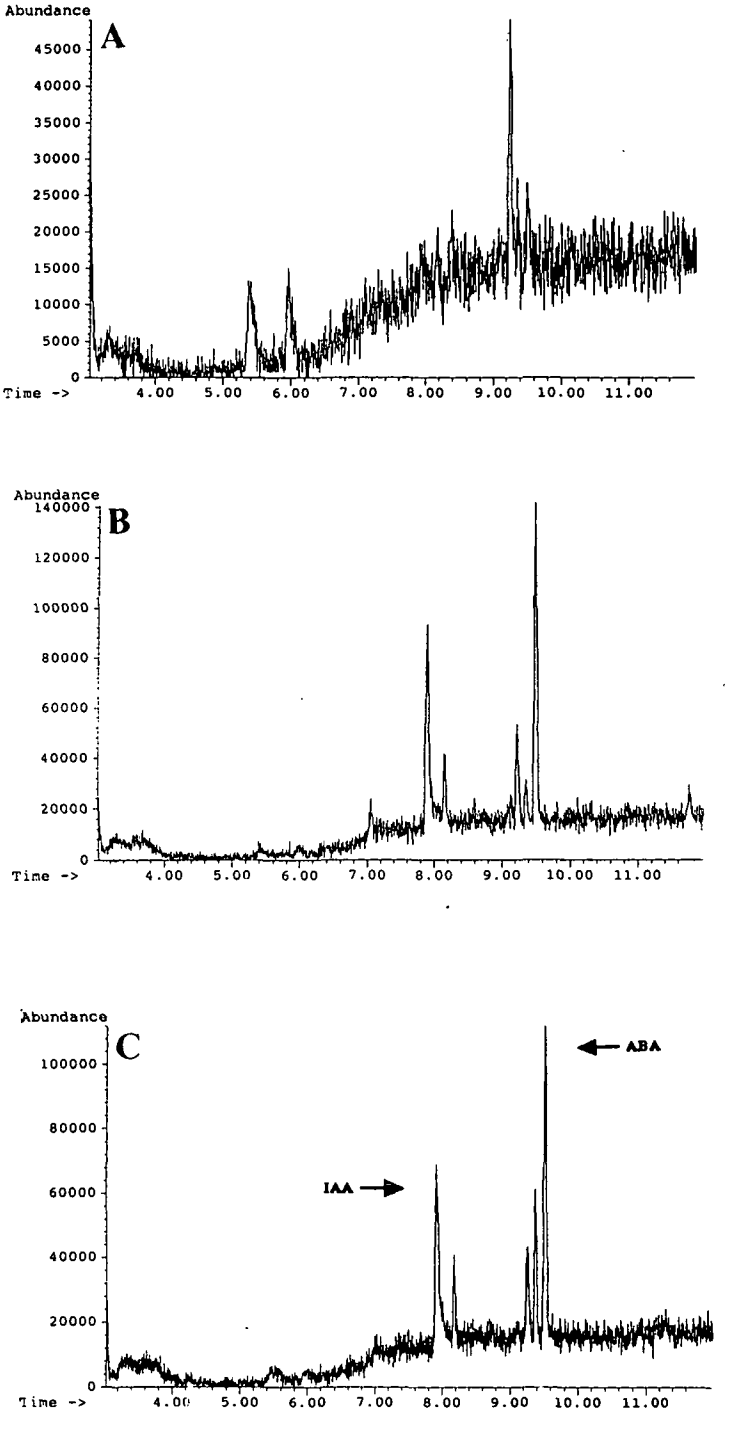

Figure 20. Comparison of GC/MS-SCAN chromatograms for determination of degree of purification using stage 9 whole ovules spiked with 1 ng/μL (+)-ABA (**A**) None. (**B**) Nylon filtering. (**C**) Nylon filtering + preparatory C_{18} column. Time is in minutes.

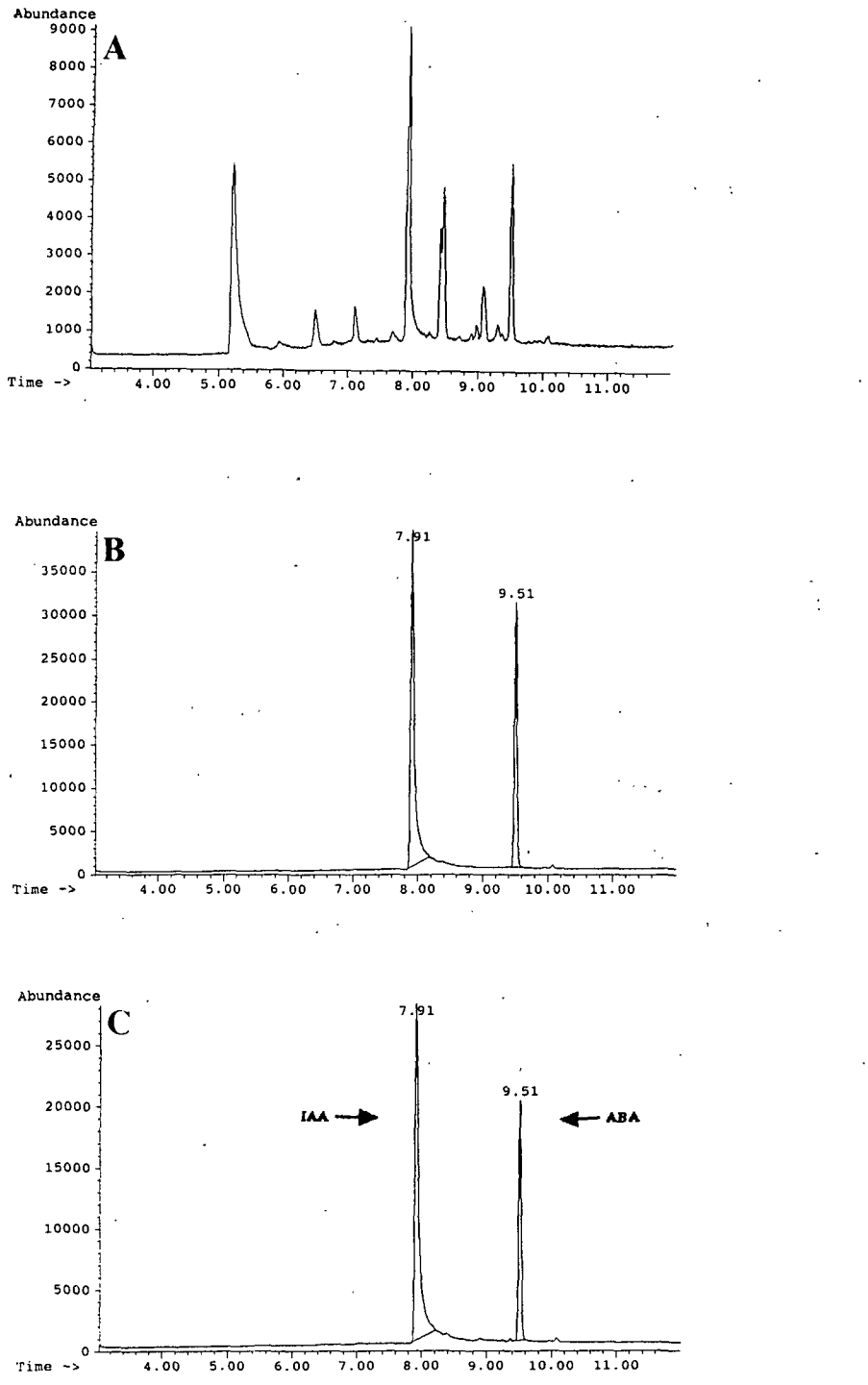

Figure 21. Comparison of GC/MS-SIM chromatograms for determination of degree of purification using stage 9 whole ovules spiked with 1 ng/μL (+)-ABA (**A**) None. (**B**) Nylon filtering. (**C**) Nylon filtering + preparatory C_{18} column. Time is in minutes.

Table 5. Comparison of GC/MS standard and sample m/z 190/162.

Statistic	ABA Standard	Megagametophyte Samples (Stages)		
		4	8B	9
Mean	2.8	2.6	2.7	3.2
Sample Size	28	6	6	10
Std. Dev.	0.38	0.58	0.63	0.20
95% C.I.	3.8-2.6	3.2-2.0	3.3-2.0	3.3-3.0

Successive Approximation for Determining Purification Requirements for the ABA Indirect ELISA

Table 6 illustrates the results of two separate successive approximation trials for determining the necessary level of purification for the indirect ELISA analysis of ABA. Whole Stage 9 ovules contained the greatest amount of contaminants, i.e., lipids, proteins, etc., and were, therefore, selected so as to test the selected protocols under the severest conditions. In Trial No. 1, ELISA estimates between crude extract and nylon filtering were statistically equivalent at $\alpha = 0.05$ and $\theta = 10\%$ of mean value for no purification (none). The sample prepared using a nylon filter + preparatory C_{18} column was lost prior to analysis due to spillage. In Trial No. 2, using a different collection of Stage 9 ovules, there were no significant differences between crude extract, nylon filtering, or nylon filtering + preparatory C_{18} column at $\alpha = 0.05$ and $\theta = 10\%$ of mean value for no purification (none). Although these results suggest that the use of crude extracts in ELISA would result in accurate ABA estimates, nylon filtering was chosen as the purification method of choice in order to maintain

Table 6. Successive approximation of the ABA indirect ELISA.

Trial No.	Degree of Purification	ELISA Results, ng/g dry-weight				Statistical Analysis, Equivalent?[a]
		Sample Size	Mean	Standard Deviation	95% Confidence Intervals	
1	None	3	164	97	---	Yes
	Nylon Filter	3	141	26	53	
	Nylon Filter + Preparatory C_{18}	3	---	---	---	
2	None	3	71	8.3	20.5	Yes
	Nylon Filter	3	69	4.6	11.4	
	Nylon Filter + Preparatory C_{18}	3	81	20.4	50.8	

a: Schuirmanns' equivalence method for two sided t-tests (155). Yes indicates that all three levels of purification were statistically equivalent at an $\alpha = 0.05$ and $\theta = 10\%$ of mean value for no purification (none).

consistency between the ELISA and GC/MS analysis protocols. For the 1992/1993 ABA analysis of loblolly pine zygotic tissue extracts (see Chapter Three), the percent recovery of ^{3}H-ABA after nylon filtering during was 81 ± 1% (95% confidence interval; sample size = 545).

ABA INDIRECT ELISA

This section will detail results for the following aspects of the indirect ELISA: optimization conditions, determination of sensitivity, estimates for precision and accuracy, and checks for interference.

Optimization of the ABA Indirect ELISA

Reagents

Checkerboard titrations were used to determine the optimal dilution for routine use of the ABA-BSA and anti-ABA mAb as detailed in the Materials and Methods Section. For each newly synthesized batch of ABA-BSA or purchased anti-ABA mAb, a titration trial was run to determine new optimal conditions as new reagents usually vary somewhat in concentration. For example, Fig. 22 illustrates the final absorbances at 450 nm of a titration plate developed using final anti-ABA mAb concentrations of 9, 8, 7, 6, and 5 ng/100 µL versus ABA-BSA concentration dilutions of 1:20,000, 1:50,000, 1:100,000, 1:150,000, and 1:200,000. The GAM-biotin conjugate and strept-HRP were used at four times and three times the manufacturers' recommended concentration, respectively. The results suggested that the optimal conditions were approximately 7 ng/100 µL anti-ABA mAb and a 1:50,000

MONOCLONAL ANTIBODY SERIES DILUTION (ng/100 µL)

ABA-BSA SERIES DILUTION		9		8		7		6		5		
		1	2	3	4	5	6	7	8	9	10	11	12
	A												
1:20,000	B		1.66	1.77	1.62	1.57	1.42	1.43	1.31	1.33	1.19	1.22	
1:50,000	C		1.60	1.60	1.55	1.47	1.37	1.39	1.24	1.19	1.01	1.50	
1:100,000	D		1.31	1.32	1.23	1.19	1.13	1.03	1.03	0.99	0.84	0.85	
1:150,000	E		0.79	0.75	0.77	0.75	0.63	0.64	0.58	0.54	0.45	0.42	
1:200,000	F		0.61	0.68	0.71	0.66	0.61	0.61	0.52	0.50	0.40	0.42	
	G												
	H												

Figure 22. Example of a checkerboard titration to optimize ABA-BSA and Anti-ABA mAb concentrations.

dilution of ABA-BSA since the final absorbance fell within the designated range of 1.3-1.5 and did not increase significantly with further increases in ABA-BSA concentration (saturated conditions). Under these conditions, the strept-HRP conjugate was optimized by titration from 1:2,000 to 1:10,000 dilution. As shown in Table 7, a concentration of 1:6,000 resulted in an absorbance of 1.33 and was considered optimal.

Incubation Time and Temperature

After optimization of the assay, incubation times and temperatures were shortened and increased, respectively, so as to reduce assay time and variability associated with temperature fluctuations in the laboratory (which were significant at times). Optimal incubation times and temperatures were 45 minutes and 37°C for blocking, and 90 minutes

Table 7. Optimal concentration of streptavidin-poly-20HRP.

Strept-HRP Dilution	Mean Absorption @ 450 nm (n = 3)
1:2,000	1.759
1:3,000	1.628
1:4,000	1.467
1:5,000	1.372
1:6,000	1.334
1:7,000	1.258
1:8,000	1.277
1:9,000	1.160
1:10,000	1.170

and 37°C for anti-ABA mAb, GAM-biotin conjugate, and strept-HRP. These changes in incubation conditions had no major effect on the standard curve or sensitivity of the assay, and these "optimized conditions" were used for all subsequent work.

Sensitivity and Linear Range of the ABA Indirect ELISA

The sensitivity of the indirect ELISA was determined by performing a dilution series of ABA standards from 800 to 0.005 pg/100 µL and creating a linear standard curve by logit transformation as described in the Material and Methods Section. A standard ABA curve was included in every plate to correct for plate-to-plate variation. The linear range was determined using the correlation coefficient (r^2) to ascertain the "best fit" linear equation through the data. Sensitivity was found to vary slightly as a function of the daily operating conditions. The sensitivity was estimated at the mean absorbance yielding a lower (-)95% confidence interval of B_o (17). As an example, Table 8 shows the mean absorbance results at

Table 8. Sensitivity trial for the ABA indirect ELISA.

ABA, ng/100 µL	Mean Absorbance @ 450 nm (n = 3)
400	0.112
7.9	0.111
2.5	0.134
0.79	0.190
0.25	0.360
0.079	0.456
0.025	0.731
0.0079	0.877
0.0025	0.885[a]
0.00079	0.954
0.00025	0.973
0 (B_0)	1.023

a: Sensitivity of indirect ELISA.

450 nm for a sensitivity trial. The -95% confidence limit for B_0 was 0.902, so the sensitivity of the trial was 0.0025 ng/100 µL or 2.5 pg/100 µL. For all the indirect ELISA performed in the course of this work, sensitivity ranged from 2.5 to 7.9 pg/100 µL with a linear range from 2.5 to 7900 pg/100 µL. Logit-transformed linear regressions with coefficients (r^2) of 0.92-0.99 were considered acceptable for quantitation of ABA.

Precision and Accuracy of the ABA Indirect ELISA

Precision in ELISA Alone and in Combination with Purification Protocols

Precision was estimated for the ELISA procedure alone, as well as for the purification and ELISA procedures in combination. The results were useful for determining what analytical protocols produced the principal variation in the assay and facilitated correction where necessary. The results are shown in Table 9 for ELISA alone and in Table 10 for Purification and ELISA together.

Indirect ELISA Trial Nos. 1 and 2 (Table 9), using an ABA standard of 0.25 ng/100 μL, gave an indication of variability in the absence of plant tissues, as well as an estimate of accuracy. The coefficient of variation ([mean/standard deviation] x 100%) was approximately 27.4% for both trials, indicating a moderate-to-high level of variability with the ELISA estimates (less than 10% is preferred (16)). In Trial No. 1, the estimate was not statistically equal to the standard (0.25 ng/100 μL). In Trial No. 2, the estimate as statistically equal to the standard. In Trial No. 3, Stage 9 ovules were used as this tissue contained the greatest concentrations of contaminants as lipids, starches, and proteins (dry-weight accumulation). The coefficient of variation for Trial No. 3 was similar to that for Trial Nos. 1 and 2.

Trial Nos. 4 and 5 also used Stage 9 ovules to estimate overall variability in the purification and ELISA protocols. As Table 10 illustrates, the coefficients of variation were slightly higher (31.3%) than those determined for the ELISA protocol alone (28.1%) indicat-

Table 9. Variation in the ABA indirect ELISA only.

Trial No.	Indirect ELISA Only, ng/100 µL or ng/g dry-weight				
	Sample Size	Mean	Standard Deviation	95% Confidence Intervals	Coefficient of Variation, %
1[a]	17	0.176	0.055	0.025	27.8
2[a]	23	0.281	0.076	0.033	27.0
3[b]	20	132	39	18	29.5
				Average:	28.1%

Table 10. Variation in combined purification and the ABA indirect ELISA.

Trial No.	Indirect ELISA, ng/g dry-weight				
	Sample Size	Mean	Standard Deviation	95% Confidence Intervals	Coefficient of Variation, %
4[b]	20	103	36	17	35.0
5[b]	20	101	28	13	27.7
				Average:	31.3%

a: (+)-ABA standard of 0.25 ng/100 µL.
b: Stage 9LL whole ovules.

ing that a majority of the variation in the analytical methods came from the ELISA while relatively little variability resulted from the purification methods. There was also very good agreement in mean results for ABA content in Stage 9LL tissue between Trial Nos. 3, 4, and 5.

Accuracy and Checks for Interference

Additivity. The results of the additivity experiment are shown in Table 11 for early (Stage 3), middle (8B), and late (9) developed megagametophytes. As the data shows for each stage of development, a change in extract dilution, i.e., full, half, or quarter, did not statistically alter the final ABA estimate. Therefore, the test of additivity suggested that the ELISA estimates were accurate and that there were no significant interferences from the plant extract within the dilution range tested. The majority of tissues were diluted within this range, i.e., from full to one-eighth strength.

Parallelism. The test for parallelism was conducted on early (bulked Stage 4 and 5) and late (bulked Stage 9) megagametophytes so as to cover the range of developmental stages. The results are illustrated in Figs. 23A and 23B, respectively. For all tissues, the slopes were approximately one (1) and the lines generated for samples containing plant extracts were essentially parallel to the standard curves. Therefore, the test for parallelism indicated that the tissue extracts were free of major interferents, and that the indirect ELISA estimates were accurate.

Table 11. Effects of dilution ratio on the ABA indirect ELISA estimates.

Stage of Tissue Development	Dilution Ratio	ELISA Results, ng/ g dry weight				Statistical Analysis, Equivalent?[a]
		Sample Size	Mean	Standard Deviation	95% Confidence Limits	
3 (early)	Full	3	685	48	120	Yes
	Half	3	597	14	34	
	Quarter	3	624	111	276	
8B (middle)	Half	3	895	109	173	Yes
	Quarter	5	867	165	204	
	Eighth	5	833	123	152	
9 (late)	Half	5	564	87	108	Yes
	Quarter	5	542	27	33	
	Eighth	5	485	50	62	

a: Schuirmanns' equivalence method for two-side t-test (155). Yes indicates statistical equivalence at $\alpha = 0.05$ and $\theta = 10\%$ of half dilution estimate.

GC/MS-SIM

This section details the results for the following aspects of the GC/MS analyses: sensitivity and range of linearity, determination of enriched stable isotope $^2H_6$-ABA concentration, development of the standard curve, and validation of the indirect ELISA.

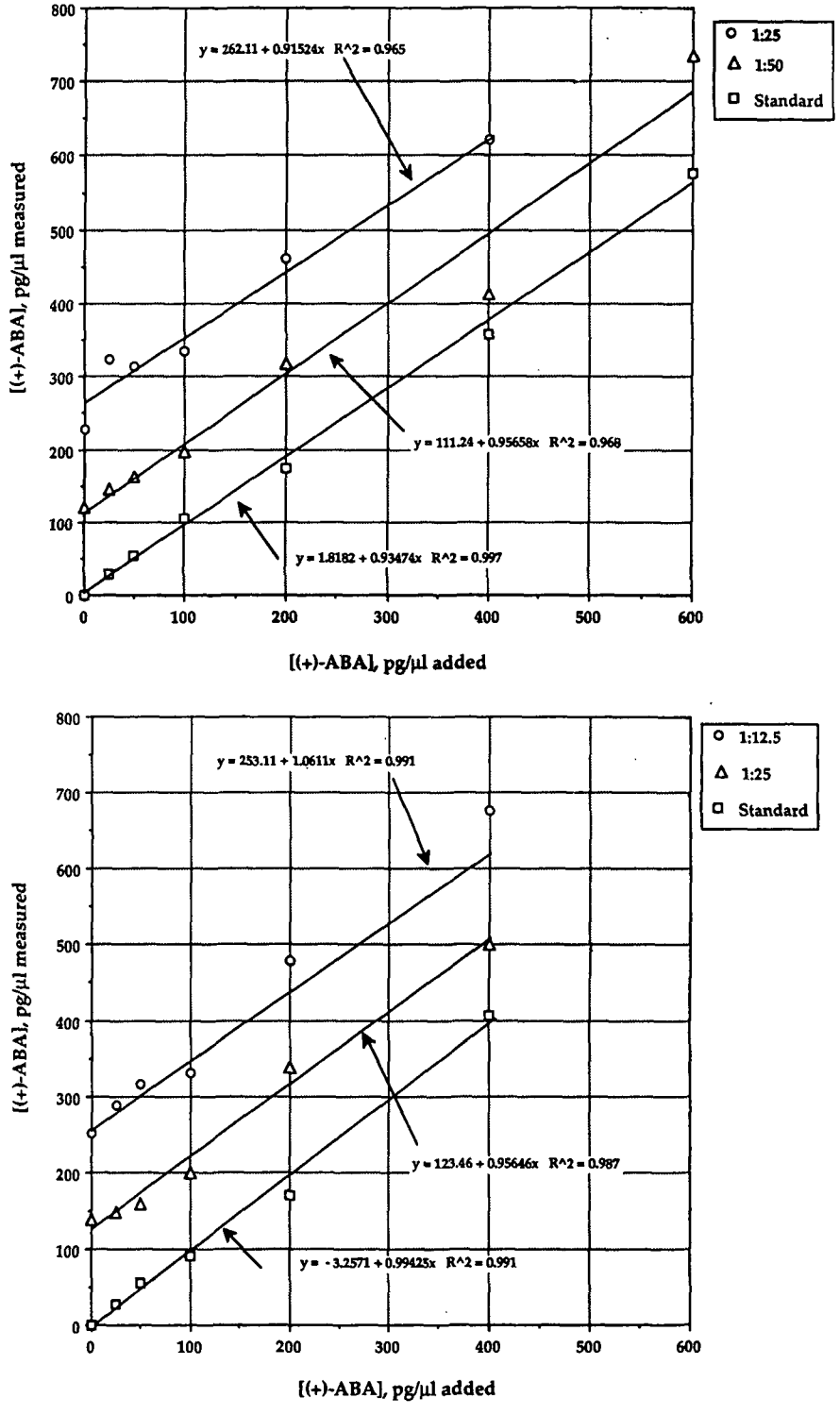

Figure 23. Test of parallelism in the ABA ELISA for (**A**) Early (Stage 4 & 5) megagametophytes. (**B**) Late (Stage 9) megagametophytes.

GC/MS Sensitivity and Range of Linearity

Sensitivity of the GC/MS was estimated as 25 pg/μL (±)ABA by plotting (±)ABA concentration versus PAR of m/z 190/194 (Fig. 24). Linearity was apparent over an (±)ABA concentration range of 25 to 1000 pg/μL with a correlation coefficient of 0.991 from the linear regression.

Determination of Enriched Stable Isotope $^2H_6$-ABA Concentration

The concentration of the enriched stable isotope ABA standard was also determined by plotting (±)ABA concentrations versus the PAR of m/z 190/194 as shown in Fig. 24. At PAR = 1, the $^2H_6$-ABA concentration was assumed equaled the (±)ABA concentration in the standards. Using linear regression analysis, the linear relationship between the PAR 190/194 and [(±)-ABA] was determined. The diluted $^2H_6$-ABA concentration was subsequently calculated as 251 ± 22 pg/μL (95% C.I.) instead of 200 pg/μL, as originally estimated. The concentration ratio (CR) between ABA and $^2H_6$-ABA on the calibration curve was consequently readjusted to range from 3.98:1 to 1:10 instead of 5:1 to 1:8.

Development of a Standard Curve Using Isotope Dilution

The GC/MS calibration curve was constructed by plotting the CR of ABA:$^2H_6$-ABA versus the PAR of m/z 190/194 as shown in Fig. 25. The PAR values calculated for the plant extracts, as measured by GC/MS, were used to determine the CR which, when multiplied by the amount of $^2H_6$-ABA added, equalled the amount of (±)ABA (pg/μL) in the sample (Equation 4).

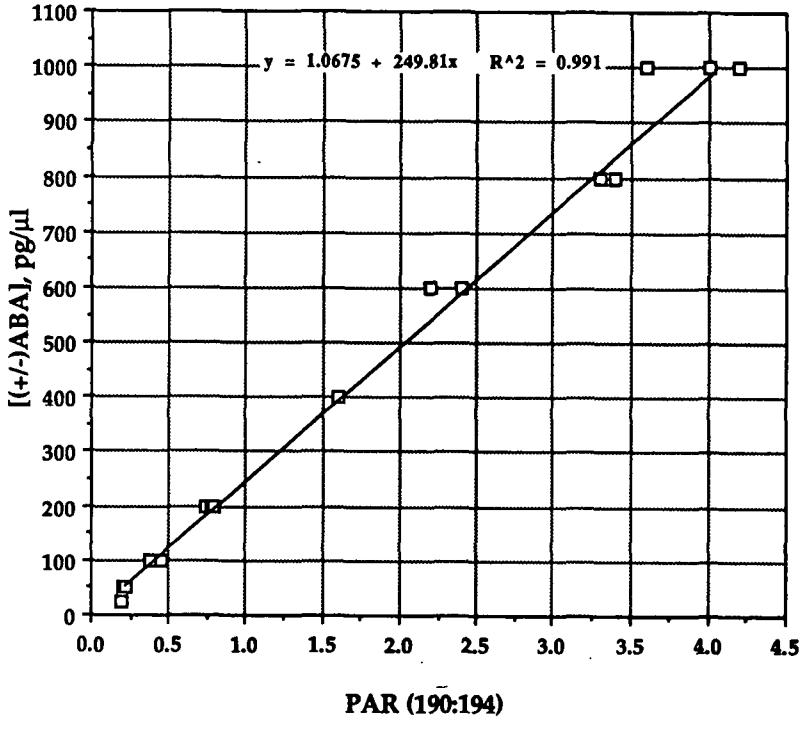

Figure 24. Plot of (±)ABA concentration versus peak area ratio (PAR) of m/z 190/194.

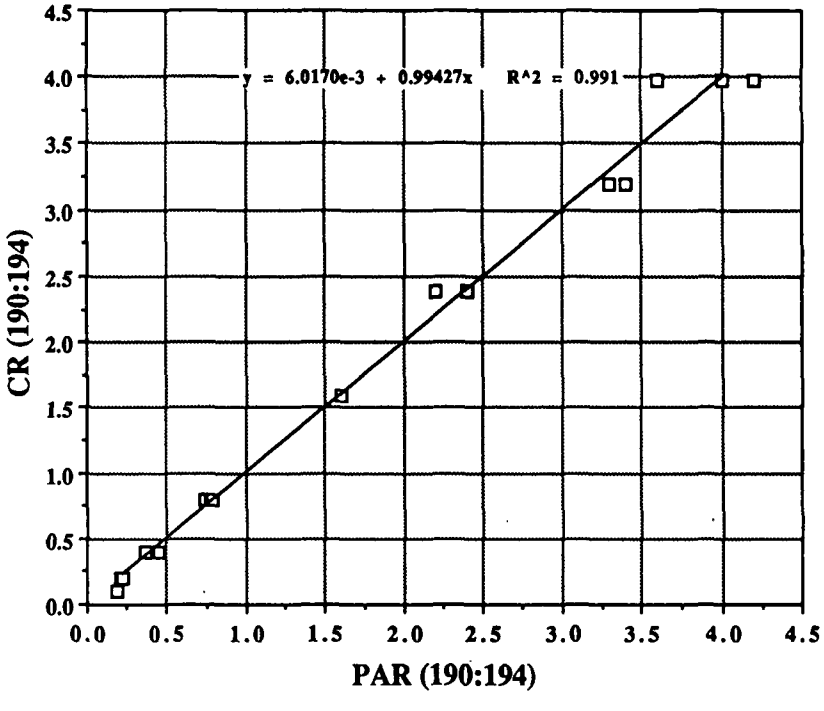

Figure 25. Calibration curve of concentration ratio (CR) versus peak area ratio (PAR) for determining endogenous ABA concentrations.

Validation of the ABA Indirect ELISA

Validation of the indirect ELISA was performed on early (Stage 4), middle (Stage 8B), and late (Stage 9) megagametophytes to cover a range of developmental stages. There was insufficient tissue available for validation of the ELISA for embryo or suspensor extracts; therefore, the assumption was made that validation of the megagametophytes extracts at disparagate stages would serve to verify the assay for embryo and suspensor extracts. Mega-gametophytes from different mother trees were bulked at all stages so as to obtain sufficient mass for analysis, as well as to obtain results applicable to more than one mother tree.

The results of the validation tests are shown in Table 12 as (±)-ABA for all three developmental stages. ELISA and GC/MS results for Stage 4 and 8B tissues were found to be significantly equivalent at $\alpha = 0.05$ and $\theta = 20\%$ of ELISA estimate using Schuirmann's two-sided t-test for equivalence (155). There was not enough data for the Stage 9 tissue to show equivalence at $\alpha = 0.05$, but at $\alpha = 0.15$ (75% C.I.) and $\theta = 20\%$ of ELISA estimate, ELISA and GC/MS results were equivalent. Dr. Pharis (156) stated that the validation at Stage 9, although not statistically equivalent, was very good and acceptable for proving the accuracy of the indirect ELISA. Therefore, validation of the indirect ELISA estimates for early, middle, and late developed tissues were considered to be accurate as assessed by the definitive physico-chemical analytical method, GC/MS.

Table 12. Validation of the ABA indirect ELISA via GC/MS.

Stage of Tissue Development	Analysis Method	ELISA and GC/MS-SIM Results, [(±)-ABA], ng/g dry weight				Statistical Analysis, Equivalent?[a]
		Sample Size	Mean	Standard Deviation	95% Confidence Limits	
4 (early)	ELISA	13	344	50	30	Yes
	GC/MS	6	377	44	46	
8B (middle)	ELISA	13	153	18	11	Yes
	GC/MS	6	169	10	11	
9 (late)	ELISA	19	270	52	25	No[b]
	GC/MS	10	330	15	11	

a: Schuirmanns' equivalence method for two-side t-tests (155) at $\alpha = 0.05$ and $\theta = 20\%$ of ELISA estimate.
b: Equivalent at $\alpha = 0.15$ and $\theta = 20\%$ of ELISA estimate.

DISCUSSION

Methodology was developed for the accurate and sensitive quantitation of ABA levels in loblolly pine zygotic tissues representing different developmental stages over the course of embryogenesis. This included development of appropriate purification techniques for extracts of these tissues and an indirect ELISA amplified using a streptavidin-biotin complex containing multiple HRP enzymes for improved sensitivity towards ABA. Techniques for validating the ELISA using a physico-chemical method, GC/MS-SIM and determination of accuracy using successive approximation, additivity, and parallelism were also required.

EXTRACTION AND PURIFICATION

Extraction

The literature suggested methanol to be slightly more effective than acetone in extracting ABA from plant tissues and that, in general, aqueous methanol (10-20% water) solutions are superior to either acetone or water alone (13). Methanolic solutions also limit enzymatic hydrolysis of ABA-conjugates to ABA at high pH, as well as methylation of carboxylic groups at low pH (16,23,157). Also based on literature reports, overnight extraction at 4°C in the dark under pre-purified nitrogen gas, appeared to be the conditions most likely to minimize oxidation and isomerization of *cis*-(+)-ABA. Despite the fact that it has not been proven to prevent the oxidation of ABA (13), BHT (25 mg/L) was added to the extraction solvent as a precautionary measure. Solvent to tissue ratio (mL:g dry-weight) used

for this study varied from 500:1 to 2000:1 depending on the amount of available tissue. At this ratio, multiple extractions were considered unnecessary.

Purification

Suitable clean-up of plant extracts is critical for the ELISA, as it is for all quantitative techniques, but extraneous compounds do not necessarily need removal unless they are a potential source of interference. The most pertinent sources of interference in immunoassays are: 1) compounds, similar in structure to the antigen, that cross-react strongly with the antibody; 2) excessive amounts of compounds which exhibit weak cross-reactivity with the antibody; 3) compounds that denature the antibody; 4) factors that prevent the binding of the antigen to the antibody; and 5) factors that impair the quantitation step, i.e., inhibit the reporter enzyme reaction (27). Therefore, a compound with identical chromatographic properties, but different immunological properties, to the antigen would not be considered a contaminant that necessarily requires removal. There are several well-established tests for determining immunoassay validity, and it is strongly recommended that the maximum number of these tests be utilized when dealing with previously untested plant materials (27). These tests are 1) successive approximation, 2) additivity, 3) parallelism, 4) validation by other quantitative methods, and 5) immunohistograms (13,15,27,28). Successive approximation and tests of additivity and parallelism will be discussed in this section. Validation by other quantitative methods will be discussed in the GC/MS Section.

Successive approximation (Table 6) performed on the most heavily contaminated tissue, i.e., greatest dry-weight accumulation (lipids, starches, and proteins), showed that no

purification (crude extract) resulted in ABA estimates that were statistically equivalent (α = 0.05) to extracts purified by nylon filtering and nylon filtering + preparatory C_{18} column. Although not necessary for accurate ELISA analysis, nylon filtering was chosen for routine clean-up of the plant extracts for the following reasons: 1) nylon filtering removed substantial particulate from the plant extract; 2) it was the least complex purification method found to yield accurate GC/MS ABA estimates; and 3) it did not require significant additional work for the level of purification obtained. Tests of additivity (Table 11) and parallelism (Fig. 23) on early, middle, and late developing megagametophytes further supported the conclusion that nylon filtering provided adequate sample clean-up with respect to potential interferences.

Although one advantage often touted for immunoassays is their ability to accurately measure materials in crude plant extracts, this is not always the case. The literature contains many instances in which antigen-antibody binding inhibitors and non-specific cross-reactants to the antibody have presented difficulties when crude or under-purified plant extracts were assayed (161). In a few cases, freezing (33) or filtering/centrifuging provided adequate purification (30,73,162,163,164), but in most instances, preparatory reverse-phase C_{18} chromatography (83,151), preparatory reverse-phase C_{18} chromatography + reverse-phase HPLC (25,55,165), or solvent partitioning with or without HPLC (29,52,157,159,166,167) was necessary. Also, in a few studies, conclusive validation of the immunoassay was not performed, so it is unknown whether adequate purification was accomplished. The conclusion reached is that the degree of purification is more dependent on the tissue than on the quantitative method; furthermore, and each new tissue and purification procedure must be thoroughly validated by as many of the above mentioned techniques as is possible.

Lastly, Fig. 19 illustrates that during extraction and purification, ABA-conjugates were not hydrolyzed and ABA was not methylated or degraded by the solvent and pH conditions. Therefore, extraction of pine seed tissues with 80% methanol containing 25 mg/L BHT adjusted to pH 7.0-7.5, and purifying the extracts by passage through 0.45 μm nylon filters did not alter the post-harvest ABA concentration or cause rearrangement of ABA conjugates. Tissue extracts treated in this manner were also free of immunological interferences.

ABA INDIRECT ELISA

The indirect ELISA was optimized to use as little of the anti-ABA mAb (7 ng/well) as possible. In comparison, other indirect ELISAs for ABA report using 0.8 μg (30), 0.5 μg (151), or 0.3 μg/well (54) of the same mAb. The advantages of using smaller amounts of mAb per assay include the obvious cost savings, but more importantly, this modification shifted the assay's standard curve so as to provide higher sensitivities. That is, the lowest detectable concentration of ABA was reduced into the picogram range, making the assay sensitive enough for routine quantitation of loblolly pine zygotic tissues. The disadvantage of increasing the ELISA sensitivity in this fashion is that its ability to discriminate between two concentrations of ABA, or the assay's precision, is reduced due to flattening of the slope of the standard curve (17,37). Increasing the equilibrium constant (K) for anti-ABA mAb binding could also have increased the sensitivity of the ELISA. K can be increased by increasing incubation time and optimizing the pH (17). Although increasing the incubation temperature (37°C) generally improves K, this is not always the case as some antibodies, i.e.,

"cold antibodies", may only react at RT or lower (4°C) (17,37). This was the case in a study by Weiler who found a 16% increase in antigen-antibody binding at 4°C relative to RT in an ABA RIA (164). Norman et al. (30) found that addition of 4% PEG to the anti-[mouse antibody]-alkaline phosphatase conjugate reaction dramatically accelerated the immune reaction and doubled the final absorbances; incubation time was reduced from 4 to 1.5 hours, mAb concentration and well-to-well variation were reduced, and assay sensitivity improved. As noted in Table 2, the incubation temperatures in this study were increased, with the exception of the TMB color reaction, which remained at RT since the horseradish peroxidase was not much influenced by these temperatures (149). Incubation times were shortened to reduce overall assay time. The overall result of these changes was that there was no improvement in sensitivity of the assay; however, reducing the incubation times might have decreased K. In general, most of the steps for ELISA/RIAs reported in the literature were conducted at RT, except for the substrate reaction which was sometimes performed at 37°C (30,33,39,54,151,160,162,166). In a few cases, all incubations were conducted at 37°C (29,55,165). Table 13 compares the sensitivities and precision of this amplified indirect ELISA to other assays reported in the literature. The sensitive indirect ELISA developed here is also reported in Results Section, and its precision is shown in Tables 9 and 10.

As shown, the low end sensitivity of the amplified indirect ELISA using an avidin-biotin interaction (168) was lower than all reported assays, except for that described by Harris and Outlaw (18). This highly amplified system also has a usable range that is somewhat broader than most. Unfortunately, the precision of the amplified indirect ELISA described herein was worse than those reported in the literature. This is undoubtedly due to the very

Table 13. Comparison of usable range and variation of the developmental ELISA to the literature.

Assay Type	Temperature, °C	Usable Range, pg/assay	Coefficient of Variation, %	Reference
This assay	37	2.5-7,900	31	---
RIA	RT	8-792	3-8	163
ELISA	RT	26-7,900	2.9	161
ELISA	RT	25-250,000	7.5	167
ELISA	RT	26-13,200	4.5	166
Indirect ELISA	RT	20-500	14-18	151
Indirect ELISA	RT	5-250	15	54
Indirect ELISA	RT	20-400	13-16	30
Amplified Indirect ELISA	RT	0.026-13	5-10	18
Indirect ELISA[a]	37	10-1,000	---	55

a: The assay developed herein was amplified from this protocol using strept-HRP.

low usage of antibody, broadness of the usable range, and to operator error, i.e., pipetting, incubation times, etc. Large samples sizes were required to make up for this poor precision (17), but due to the assays design, i.e., minimal purification and short overall assay time, large samples sizes could be run with little procedural effort.

VALIDATION OF THE ABA INDIRECT ELISA BY GC/MS

The major criticism of ABA immunoassay work in the past is that few independent checks have been made on the accuracy of the assay. Without independent analysis of ABA by physico-chemical methods, preferably using GC/MS-SIM, the RIA/ELISA results must be

considered suspect and may well be unusable in certain instances. Although tests for additivity and parallelism can provide sound evidence for the absence of interferences, immunoassay results can not be considered reliable without proper checking of quantitative estimates by a second method. This said, only a handful of studies have provided corroborative evidence using physico-chemical technique. Only five reported studies used the most credible method, GC/MS (33,151,159,163,169), two used GC/ECD (162,165), several used less reliable HPLC-UV (30,54) or HPLC-indole-α-pyrone fluorescence (83), but the majority used no validation at all or stated that the immunoassay was previously validated by other researchers (using different plant species and/or tissues) (18,25,27,73,78,158,166,167,170).

The amplified indirect ELISA for ABA presented here has been validated by tests of additivity and parallelism, as discussed earlier, as well as by GC/MS-SIM providing conclusive evidence for the accuracy of the ABA estimates. As recommended by Pharis (156), extracts from early-, middle-, and late-developed tissues were checked by ELISA and GC/MS-SIM to determine whether ELISA interfering substances or cross-reactive immunoactive substances might be present at one developmental stage, but not at an other. Such a thorough validation has never been performed in the literature. As the results demonstrated (Table 12), the two methods gave statistically equivalent estimates for early- and middle-developed megagametophyte tissues at $\alpha = 0.05$ and equivalent estimates for late-developed tissues at $\alpha = 0.15$. Pharis (156) has stated that these results should be considered very good. The only limiting assumption in this validation is that megagametophytic and embryo tissues likely contain identical compounds that might have acted to interfere with the assay. Percent differences in ABA estimates during immunoassay validations from the literature are

compared to the assay developed herein (Table 14). The percent differences obtained between the immunoassay and the validation method varied from 2-11%. Therefore, the differences found for Stage 4 and Stage 8B were considered acceptable, while the difference for Stage 9 tissue (22%) was greater than any found in the literature.

Table 14. Comparison of the developmental ELISA to the literature in regards to differences between immunoassay and validation results.

Assay Type	Validation Technique	Percent Difference	Reference
This assay (ELISA)	GC/MS	Stage 4 - 10% Stage 8B - 10% Stage 9 - 22%	---
RIA	GC/MS	2%	163
ELISA		5%	159
ELISA		25%[a]	151
RIA	GC/ECD	3%	33
ELISA		4%	162
ELISA		10%	165
ELISA	HPLC	5%	54
ELISA		11%	30

a: The large difference was caused by degradation of the very low ABA concentration standards.

CONCLUSIONS

The indirect ELISA for ABA, developed using Idetek's commercially available monoclonal antibody (38) and amplified using a streptavidin-biotin-multiple horseradish peroxidase detection system, has been shown to give reliable and very sensitive estimates for ABA while conserving mAb usage. Since only nylon purification was required for clean-up of pine tissue extracts, sample sizes could be very small, and as little as 0.1-0.05 mg of tissue was sufficient for a single ABA extraction with 10-13 replications. Furthermore, as many as 400 assays could be easily carried out in a day by one person versus only 10-15 assays on the GC/MS. Therefore, this quantitation method was utilized for investigating the distribution of ABA in loblolly pine zygotic tissues undergoing embryogenesis during the 1992 and 1993 growing seasons.

CHAPTER THREE - QUANTITATION OF ABA IN LOBLOLLY PINE ZYGOTIC TISSUES

INTRODUCTION

The goal of this research was to measure the change in endogenous ABA concentrations in embryo, megagametophyte, and suspensor tissues during zygotic embryogenesis in loblolly pine. To accomplish this goal, the following objectives were performed: 1) develop appropriate techniques for collecting, extracting, and storing seed tissues from loblolly pine cones; 2) develop appropriate dissection techniques for separation of embryo, suspensor, and megagametophyte tissues; and 3) measure the ABA concentrations in the zygotic tissues (whole ovule, embryo, megagametophyte, and suspensor tissues) representing different stages in loblolly pine embryogenesis from the 1992 and 1993 seed development seasons.

MATERIALS AND METHODS

PLANT MATERIALS AND METHODS

Plant Materials

Open pollinated loblolly pine cones (two to six) from genetically superior mother trees were obtained from seed orchards from Westvaco Corporation, Summerville, SC (one mother tree; WV_{92} [genotype 8]); Union Camp Corporation, Bellville, GA (one mother tree; UV_{92} [genotype 10-84]); and Weyerhaeuser Company, Lyons, GA (two mother trees; WA_{92} and WB_{92}) during 1992. These same mother trees were collected during 1991 for method

development only. A third set of cones were collected from Weyerhaeuser during the 1993 season (WA_{93}) from one week prior to fertilization to seed maturity, i.e., fully dormant seed. Dried seed (i.e., commercially processed, dried, and stored seed) from Union Camp was a mixed lot (i.e., multiple genotypes) collected by Union Camp personnel during 1992. Cones were also obtained from a seed orchard from Westvaco Corporation, Brazil, during January-February, 1994. Cones from U.S. locations were shipped overnight, on ice packs, on a weekly basis from early June to late October in order to obtain embryo developmental stages ranging from 1 week prior to fertilization to cone maturity. Air-dried seeds were used as an end-point for fully mature seed tissues, i.e., dormant. The cones were removed from the central portion (all sides) of the trees where exposure to the environment was most consistent during development, i.e., minimal differences in sunlight, temperature, nutrients, etc. between trees. Other materials used for dissection and tissue storage included extra-fine forceps, hemostats, a binocular microscope, a light microscope, a cryogenic freezer (-80°C), cryogenic glass ampoules, liquid nitrogen, Dewar flasks, freeze-dryer flasks, and a freeze-dryer.

Plant Tissue Methods

Tissue Dissection and Storage

The dissection and storage procedures were performed as quickly as possible so as to minimize tissue and water stress and its potential effects on endogenous ABA concentrations (19). Excessive mechanical damage during handling was also avoided to minimize artificial increases in endogenous ABA. During dissections, the microscope light source was kept as dim as possible to minimize temperature and light affects on (+)-ABA concentrations

(chemical degradation and isomerization to *trans*-ABA) (94,95).

Upon arrival, cones were immediately dried of surface moisture, wrapped in paper toweling, and stored (avoided whenever possible) in polyethylene storage boxes at 4°C in the dark for no more than 24 hours. Using a guillotine device, the upper and lower quarters of the cone were removed and discarded. A screwdriver was forced down the core of the remaining cone to split the core, and the cone halves were pulled apart. The opened cones were placed on ice packs and covered in aluminum. The seeds were extracted from the cones using fingers or forceps and transferred to a covered Magenta box, also set on cold packs. Seeds damaged by the guillotine, insects, weather, etc. were discarded.

To remove the ovule from the seed coat, a hemostat was used to carefully crack open the coat along its longitudinal seam. Gentle pressure was applied until the coat split open. Under a binocular microscope, the coat halves were pulled apart using extra fine tip forceps. The ovule was removed from the seed coat by carefully sliding one tip of the forceps under the ovule and "scooping" it out of the coat.

In a disposable plastic petri plate under the microscope, the ovule was held in place with the forceps while the nucellus was carefully removed and discarded. A longitudinal cut along the ovule was made with a clean scalpel (kept clean by dipping in water and drying between dissections), and the ovule was gently pried open using forceps to expose the suspensor and embryo tissues.

The suspensor and embryo tissues were carefully separated from the megagameto-

phyte using forceps, taking care to prevent damage. The suspensor and embryo tissues were immediately dipped in a clean drop of distilled water to remove fluids that might have spilled from the megagametophyte during dissection and opening.

The suspensors and embryos were separated into individual stages for Stage 3-9 tissues (not practical for Stage 1-2) using either a scalpel or forceps tip, and each individual tissue (embryo, suspensor, megagametophyte, and/or whole ovule) was quickly frozen by dipping in liquid nitrogen. Frozen tissues were transferred to separate glass ampoules (identified by tissue type, i.e., embryo, suspensor, megagametophyte, or whole ovule; Stage of development, i.e., 1 to 9; and mother tree, i.e., UC, WV, WA, or WB) suspended in a Dewar flask containing liquid nitrogen. Stage 1 and 2 tissues collected during 1993 were separated into embryo + suspensors, megagametophytes, and whole ovules. Tissues collected one week before, and at fertilization were kept as whole ovules since discrete structures had not yet formed.

The plastic petri plate was rotated between dissections to reduce contamination. Tissues were counted during collection so as to be able to calculate ABA on a per organ basis (Appendix 1). After all the seeds were dissected (typically 45-50 seeds per collection), the ampoules containing tissues were removed from liquid nitrogen, wrapped in several layers of Kim-Wipe to prevent tissue from escaping, transferred to a freeze-drier flask wrapped with aluminum foil to reduce light, and the tissues were lyophilized for 12 to 24 hours at RT. The tissues remained frozen during lyophilization, i.e., liquid water was not observed.

After lyophilization, the ampoules were purged of air by alternate exposure to

vacuum and pre-purified nitrogen, containing 5 ppm H_2O and 5 ppm O_2, for at least six cycles using the purging device shown in Fig. 26. The removal of oxygen prevented the possibility of oxidation of ABA during storage. The mouth of the ampoule was sealed under slight vacuum (<5 in. Hg) using a oxygen/methane torch, and the ampoules were stored at -80°C in the dark to prevent isomerization of ABA (13,16).

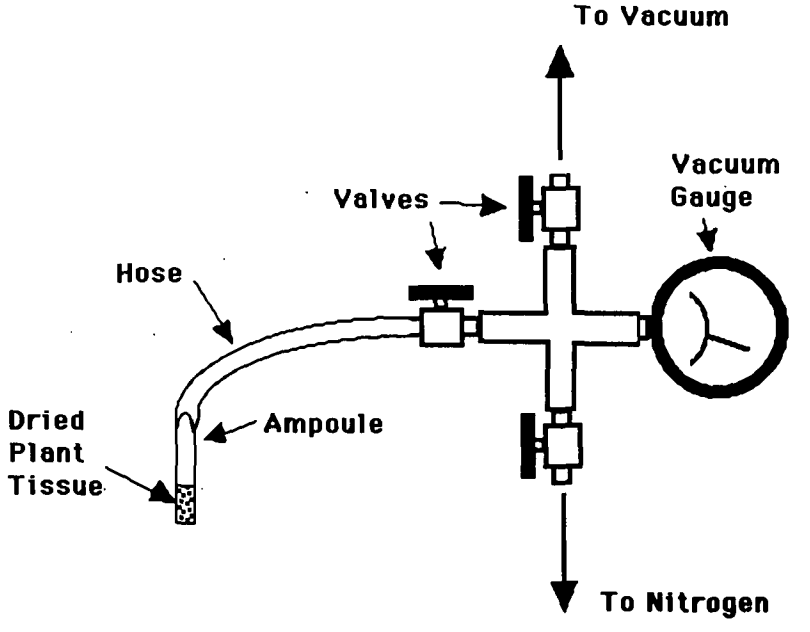

Figure 26. Purging device used to evacuate air from ampoules.

Determination of Tissue Dry-Weight and Percent Moisture Content

Embryo and megagametophyte tissues from late developmental stages in 1992, as well as embryo, embryo + suspensor, megagametophyte, and whole ovule tissues representing all stages from the 1993 developmental sequence were measured for dry-weight, and percent moisture content in order to calculate ABA on a per gram fresh weight basis (Appendix 1). The tissues used for moisture determinations were not used for subsequent ABA analysis.

Whole ovules were dissected and triplicate sets of five embryos and megagametophytes (depending on the stage of development) were used for moisture determinations. The bottom plates of glass Petri dishes were lined with damp paper towels and a microscope slide was positioned directly on top of the toweling. The moist toweling was used to maintain a high humidity which prevented the tissues from drying during collection. Slipcovers were weighed (SC), placed on top of the slide using forceps and the top of the Petri plate was replaced. During dissections, tissues were placed on the slipcover using forceps. After the collection was completed, excess moisture was blotted from the slipcovers, if necessary, the slipcovers + tissues were weighed (SC + FW), and subsequently dried in a oven at 80-85°C for at least three days. The dried tissues were allowed to cool in a desiccator prior to weighing (SC + DW). Percent moisture content was calculated using Equation 5.

$$\% \ Moisture = \frac{(FW-SC)-(DW-SC)}{(FW-SC)} \times 100\%$$

Equation 5. Determination of percent moisture content in seed tissues.

Dissection and Washing on Endogenous ABA Concentrations

Tissues from Westvaco Clones E and M (IPST Brazilian cone collection; 1/27/94) were collected and stored as outlined in the Tissue Dissection and Storage Section with the following exceptions. The tissues were collected separately as either 1) whole ovules; 2) tissues dissected, embryo + suspensors washed, as described in the Tissue Dissection and Storage Section, followed by pooling of the dissected tissues (i.e., embryo, suspensor, and

megagametophyte) in a single ampoule; or 3) tissues dissected, but not washed, and then pooled in a single ampoule. This experiment was designed to assess the effects of dissection alone and washing alone on endogenous ABA concentrations during the collections protocols.

Degradation of Endogenous ABA in Storage

Whole immature (Stages 2 to 3) ovules from Westvaco Clone M (Brazilian cone collection; 1/27/94) were collected and stored as outlined in the Tissue Dissection and Storage Section, except that no dissections or washings were performed. Ten whole ovules were transferred to each of six ampoules (total of 60 whole ovules collected), lyophilized, sealed, and stored under the described conditions. ABA was immediately quantitated from one of the ampoules using the indirect ELISA as described in Chapter Two. The remaining ampoules were opened and the tissues were analyzed for ABA at intervals over a 106 day period to assess the degradation of endogenous ABA during tissue storage.

Staging of Developing Zygotic Tissues

The system for staging zygotic and somatic embryo development has recently been described by Webb and Pullman (171). The staging performed in this thesis work was based on this system with only slight modification. In essence, the system is based on visible morphological changes in the embryo-proper and suspensor tissues during zygotic (and somatic) embryogenesis. Embryo and suspensor development has been organized into nine major stages with sub-staging in some cases. Accurate assignment of Stages 1 and 2 required the use of a light microscope as the embryos-proper were microscopic. The embryo-proper

was visible using a binocular microscope (4X magnification) from Stages 3 to 9, with Stage 3 representing the earliest point at which the embryo-proper could be practically separated from the suspensors. Estimation of the date of fertilization (±2 days) was based on the following visual events: 1) the appearance of archegonia (two to four) at the micropylar end of the megagametophyte (*pre-fertilization* event), 2) densification of the central portion of the megagametophyte as determined by a visible shift from transparency to translucency and, 3) the eventual break down of this central portion to form the corrosion cavity (*post-fertilization* event) (172). The reported Days Since Fertilization (DSF) corresponding to embryo stage of development (SOD) were recorded during collection. Assuming that a normal distribution of stages moved along the time line (DSF), each stage would predominate at any given time of cone dissection. Normal distributions for each stage were determined from the tissues collected at each collection date. For example, if during the tissue collections Stage 4 predominated at 40 DSF and Stage 6 predominated at 46 DSF, than it was assumed that Stage 5 tissue predominated at 43 DSF. Descriptive criteria for stages of *Pinus* are listed below and a graphical depiction is shown in Fig. 27:

Stage 1: Proembryos, from free nucleate to 12 cells; found mainly in the archegonium.

Stage 2: Embryos-proper (polyembryony) are distinct, translucent, and located anywhere from the micropyle extending up to three-quarters into the megagametophyte; microscopic. There is no dominant embryo-proper present. Suspensors have elongated, but have not increased radially.

Stage 3: Dominant embryo-proper present. It is white, opaque, and located in the middle to chalazal end of the megagametophyte; macroscopic. Suspensors have elongated and increased

radially.

Stage 4: Embryo-proper is similar to Stage 3, but larger longitudinally and radially and is still dome-shaped and opaque. Suspensors greatly elongated and thickened.

Stage 5: Similar to Stage 4, except the apical meristem is visible as a point on the embryo-proper. No signs of cotyledons.

Stage 6: Similar to Stage 5, except the apical meristem is more predominant. The cotyledons are barely visible and remain below the tip of the apical meristem.

Stage 7: Similar to Stage 6, except the cotyledons are distinctly visible and have elongated up to a height of, but not overtopping, the apical meristem.

Stage 8A: Similar to Stage 7, except the cotyledons have overtopped the apical meristem. The apical meristem is still visible. The embryo does not fill the entire corrosion cavity.

Stage 8B: Similar to Stage 8A, except the embryo and cotyledons are further elongated, and occasionally curved, but the tips have not come in contact. The apical meristem is still visible from above. The embryo completely fills the corrosion cavity.

Stage 9A-I: Similar to Stage 8B, except the cotyledons have curved and joined at their tips so that the apical meristem is no longer visible from any angle. Stage 9 tissue was collected and subdivided into weekly samples designated 9A to 9I since physiological and biochemical growth continued until cone maturity without obvious morphological changes.

Stage 9Δ: Seed maturity. Fully-dried seed obtained from orchard after removal from cones and air drying. These seeds represent "fully mature" seeds, i.e., dormant seeds ready for long-term storage and/or germination.

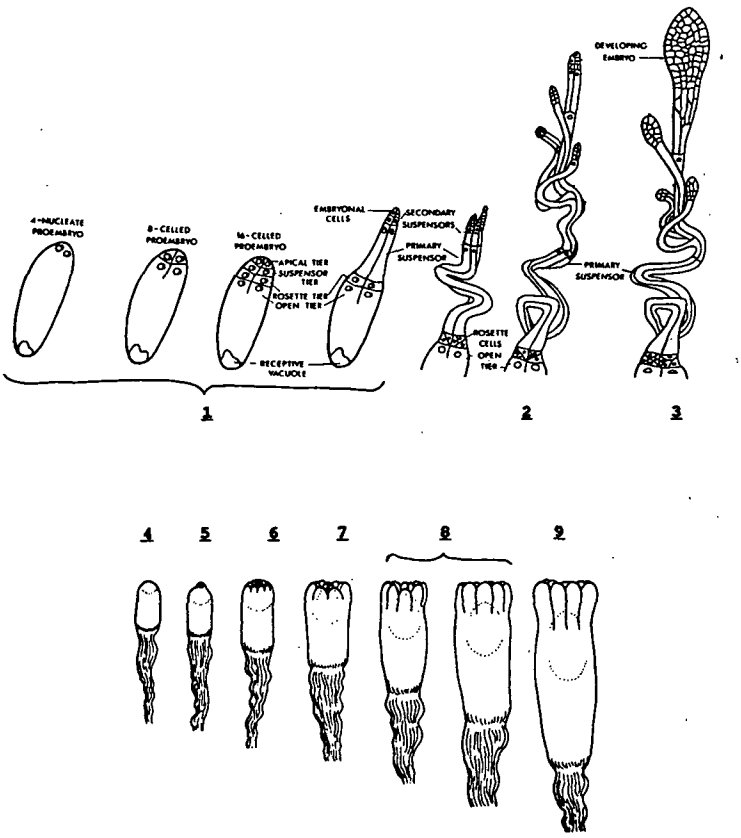

Figure 27. The morphology of the nine stages of developing embryos (171).

Determination of Endogenous ABA Concentrations During Zygotic Embryogenesis

Endogenous (+)-ABA concentrations, reported on a dry-weight basis, were analyzed in the collected tissues with respect to mother tree (UC_{92}, WV_{92}, $WA_{92\ and\ 93}$, and WB_{92}), stage of development (Stage 1-9), and tissue type (whole ovule, embryo, embryo + suspensor, suspensor, and megagametophyte) for the 1992 and 1993 growth seasons. Identical stages were bulked to increase available tissue. During 1992, embryo, suspensor, and megagametophyte tissues representing Stages 3-9 were collected and analyzed. During 1993, whole ovule (from one week prior to fertilization to Stage 3), embryo + suspensor (Stages 1-2), embryo (Stages 3-9), and megagametophyte (Stages 1-9) tissues were collected

and analyzed. ABA concentrations were measured using ELISA as described in Chapter Two. Depending on the amount of available tissue, one to four samples were prepared from each stage (0.1-0.05 mg dry-weight tissue), and typically 11 assay replicates were performed per extraction (11 to 44 samples per data point). From the dry-weight results, ABA was also calculated on a per organ, fresh-weight, and μM basis. The results (means and 95% confidence intervals) were plotted on a per dry-weight, fresh-weight, and organ basis. Spacing of the data points along the abscissa is presented with two time scales: stages of embryo development as they occurred in nature (non-linear), as well as the estimated DSF (linear). In graphs illustrating more than one mother tree, the DSF scale was used exclusively since tissues from each mother tree had slightly different rates of development, making plotting of the abscissa by stage of embryo development difficult to produce and interpret.

STATISTICAL ANALYSIS

The means and 95% confidence intervals for ABA content at each stage of zygotic development were determined and plotted versus stage of development and DSF developed. Statistical analyses for the effects of dissection and washing, as well as for degradation of ABA in storage were performed using Scheffe's Multiple Comparison. Statistical analysis for estimating the DSF for each developmental stage was performed using Students' t-tests (normal distributions). Analyses were carried out at the $\alpha = 0.05$ level.

RESULTS

PLANT TISSUE COLLECTION

This section will detail the results for the following aspects of tissue collection and storage: estimation of fertilization date, change in percent moisture content during zygotic development, effects of dissection and washing on endogenous ABA concentrations, and degradation of ABA during tissue storage.

Estimation of Fertilization and Cone Ripening Dates

Estimates for the fertilization and cone ripening dates during the 1991-1993 growing seasons are shown in Table 15. Despite weather conditions over the three years (there was considerable variation in dryness and temperature), and geographic locations of the mother trees (Table 16), the estimated fertilization dates for most of the mother trees were within three days of each other. The only exceptions were the WA_{92} and WB_{92} samples which were fertilized approximately one week earlier. The WB_{92} sample also reached cone maturity (determined at the orchard as the date when the cones would float in a pail of oil, i.e., cones reached a certain degree of dryness) approximately two to three weeks earlier than the other trees studied that year, suggesting that faster maturing seeds are not necessarily a function of the time of fertilization, but are also influenced by genetics and/or local environment.

Table 15. Estimated dates of fertilization and cone ripening for the mother trees of loblolly pine.

Year of Collection	Mother Tree (genotype)	Estimated Date of:	
		Fertilization	Cone Ripening
1991	UC_{91}	6/12	10/8
	WV_{91}	6/12	10/10
	WA_{91}	nd	10/11
	WA_{91}	nd	9/17
1992	UC_{92}	6/15	10/15
	WV_{92}	6/15	10/10
	WA_{92}	6/8	9/27
	WB_{92}	6/8	9/8
1993	WA_{93}	6/14	10/6

nd: not determined.

Estimation of Days Since Fertilization for Each Stage of Development

Table 17 compares the stage of development (SOD) of the tissues to their approximate time of appearance during zygotic development for all four mother trees during 1992 and for the WA tree during 1993. Development was quite slow immediately after fertilization, as shown by the length of time embryos remained in Stages 1 and 2. One month after fertilization, embryo development became very rapid, progressing from Stage 3 to 7 within a two week period, with almost all stages being present within cones during these collections. Development slowed down again during Stages 8A-9A, after which visible morphology no

Table 16. Genotypes and locations of the mother trees of loblolly pine.

Notation	Year of Collection	Genotype	Location of Seed Orchard		
			Latitude[a]	Approx. Elevation, m	Approx. Distance from Coast, km
UC_{91}	1991	10-68	33° 47'	12	80
WV_{91}		240	34° 59'	~ 0	40
UC_{92}	1992	10-84	33° 47'	12	80
WV_{92}		8	34° 59'	~ 0	40
WA_{92}		A	33° 49'	62	100
WB_{92}		B	33° 49'	62	100
WA_{93}	1993	A	33° 49'	62	100

a: one degree of latitude = 100 km.

longer changed.

Dry-Weight and Percent Moisture Content During Zygotic Embryogenesis

Figures 28 and 29 illustrate the change in dry-weight and percent moisture content over the course of embryo and megagametophyte development, respectively, for WA_{93} (complete data for all mother trees during 1992 and 1993 is in Appendix 1). Moisture information for the 1992 collections were only performed on Stage 9 tissue and were not included in the figure (Appendix 1). At fertilization, the whole ovule moisture level was measured as there were no discrete structures present. It was not possible to obtain accurate moistures for embryos prior to Stage 3 as they dried during the dissection process.

Table 17. Stage of development versus days since fertilization for the 1992/1993 mother trees of loblolly pine.

Stage of Development	Estimated Days Since Fertilization	
	Mean	Range[a]
1	7	0-14
2	21	14-28
3	38	33-43
4	41	36-47
5	44	41-48
6	46	43-50
7	49	45-52
8A	51	48-55
8B	56	52-60
9A	63	57+[b]
Cone Maturity (9F-I)	111	97-125

a: 95% confidence interval (n = five mother trees).
b: Stages 9B+ were collected every seven days.

Embryos and megagametophytes began drying immediately after fertilization (Fig. 29) with both tissues displaying similar curvilinear drying patterns. The megagametophyte tissues began drying earlier, and at a faster rate, than the embryos. This was not surprising given that the megagametophyte is in contact with the atmosphere to a greater degree than the embryo. It was assumed that the percent moisture content of the embryos prior to Stage 3 was identical to the megagametophyte moisture since, at those stages, the megagametophyte is in intimate contact with the small embryo + suspensor tissues and, therefore, would probably determine the moisture of these tissues. This data was used to determine the ABA

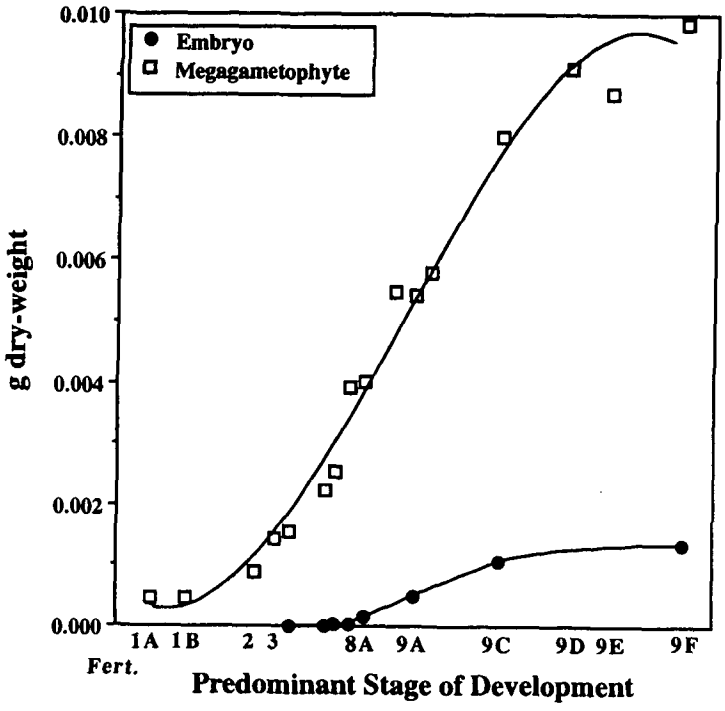

Figure 28. Dry-weight accumulation from the WA_{93} loblolly pine collection from fertilization to seed maturity.

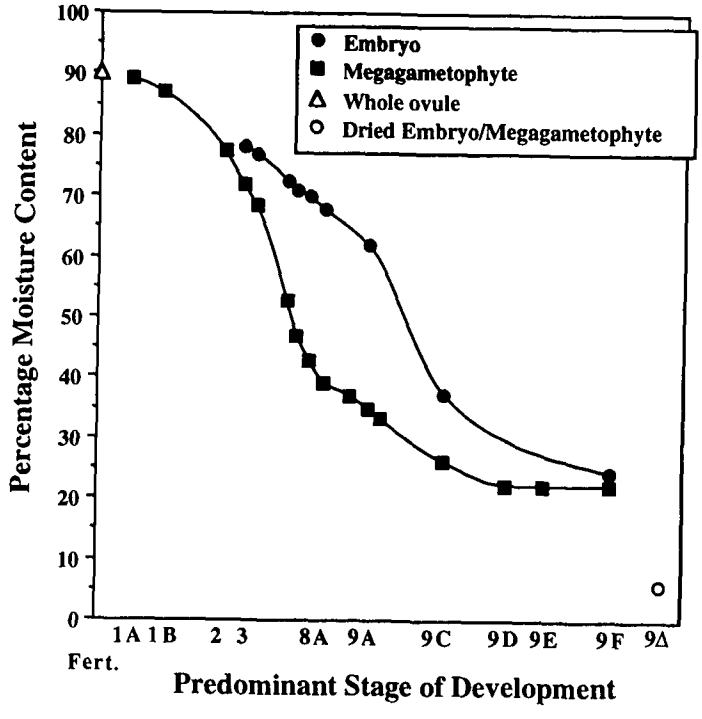

Figure 29. Percent moisture content from the WA_{93} loblolly pine collection from fertilization to seed maturity.

concentrations on a fresh-weight basis.

Effects of Dissection and Washing on Endogenous ABA Concentrations

The statistical results of the experiments to study the effects of dissecting the tissues and washing the embryo + suspensor tissues prior to storage are shown in Table 18 for two mother trees. The different letters in the statistical analysis column of Table 18 indicate a significant difference at $\alpha = 0.05$ for each mother tree. There was a significant increase (approximately 47%) in endogenous ABA concentration during the dissection of the tissues (with and without washing), i.e., opening the megagametophyte and separation of the embryo + suspensor tissues, for both trees. There was not enough statistical evidence to conclude that washing of the embryo + suspensor had any effect on endogenous ABA concentration at $\alpha = 0.05$, although the reduction of ABA in both trees would lead one to suspect that ABA was removed from the embryo + suspensor during the washing. Therefore, the ABA estimates and fluctuations from these tissues analyzed during zygotic development have been artificially increased, because of the dissection process, and are higher than the true post-harvest levels.

Degradation of Endogenous ABA during Tissue Storage

Table 19 shows the results of an experiment to determine, if and when endogenous ABA begins to degrade under the storage conditions described in the Material and Methods Section. Except for day 27, the endogenous ABA concentrations were significantly greater than those found at day 0. These results suggest that tissue for ABA analysis should be utilized immediately or at least within 18 days of initial storage, otherwise, the ABA

Table 18. Effects of dissection and washing on the ABA indirect ELISA estimates.

Mother Tree	Tissue Treatment	ELISA Results, ng/g dry weight				Statistical Analysis[a] at $\alpha = 0.05$
		Sample Size	Mean	Standard Deviation	95% Confidence Limits	
E	Whole Ovule	28	629	109	43	a
	Dissected Tissues + Washing	30	932	170	63	b
	Dissected Tissues + **No** Washing	28	1036	148	57	b
M	Whole Ovule	8	253	15	13	d
	Dissected Tissues + Washing	10	422	94	67	e
	Dissected Tissues + **No** Washing	7	564	130	120	e

a: Scheffe's Multiple Comparison Method at $\alpha = 0.05$. Statistical analysis for Mother Tree E and M are to be considered separate experiments. Different letters indicate a significant difference in ELISA estimates for ABA. Identical letters indicate that there is insufficient data available to conclude that the estimates are different.

Table 19. Degradation of endogenous ABA during tissue storage.

Days in Storage	ELISA, [(+)-ABA], ng/g dry weight				Statistical Analysis[a]
	Sample Size	Mean	Standard Deviation	95% Confidence Limits	
0	26	715	144	59	a
18	10	917	54	39	b
27	24	792	203	86	a
45	20	881	157	73	b
106	28	825	124	48	b

a: Scheffe's Multiple Comparison Method at $\alpha = 0.05$. Different letters indicate a significant difference in ELISA estimates for ABA. Identical letters indicate that there is insufficient data available to conclude that the estimates are different.

estimates will be higher than the true post-harvest level.

ENDOGENOUS ABA LEVELS IN LOBLOLLY PINE SEED TISSUES

In the following section, the results of endogenous ABA quantitation during zygotic embryogenesis will be presented: 1992 trends for four mother trees; 1993 trends for one mother tree; a comparison between 1992 and 1993 trends; and ABA contents versus rate of dry-weight accumulation and desiccation. Transformed results (means and 95% confidence intervals) are presented in Appendix 1.

Determination of Endogenous ABA in the Mother Trees

1992 Trends - Embryo

Changes in endogenous ABA levels found in developing embryos are illustrated for seeds taken from the four mother trees (WV_{92}, UC_{92}, WA_{92}, and WB_{92}) on a dry-weight basis (Fig. 30) or on a per embryo basis (Fig. 31) for samples ranging from Stage 4 to cone maturity, i.e., late Stage 9 (seed maturity for UC, i.e., 9Δ). On a dry-weight basis, all the trees showed similar trends, i.e., high concentrations of (+)-ABA early in development dropping down to the lowest concentrations at cone and seed maturity (UC). After about Stage 9C, the ABA concentrations remained relatively unchanged. A peak occurred from Stage 7 to 8B in WV_{92}, UC_{92}, and WB_{92}, but not in WA_{92}. Despite the differing sizes, these peaks indicate a significant increase in ABA, but the physiological significance is unknown as they are minor in comparison to the amount of ABA present prior to Stage 4. It is possible that WA_{92} might also have had a peak during Stage 7-8A, but it was missed between two collection dates. ABA concentrations at Stage 4 varied significantly between mother trees, from a high of about 9,000 ng/g dry-weight for WA_{92} and to a low of about 2,500 ng/g dry-weight for UC_{92}. Data for Stage 3 embryos was not included on Fig. 30 as there was insufficient mass available to weigh accurately. Variability, shown by the 95% CI, was much higher for the early tissues than the late tissues, and was most likely due to error in weighing. In some cases, less than ten embryos were available, which made weighing on a microbalance difficult (<0.05 mg dry-weight). However, bulking of the tissues may also have had an effect.

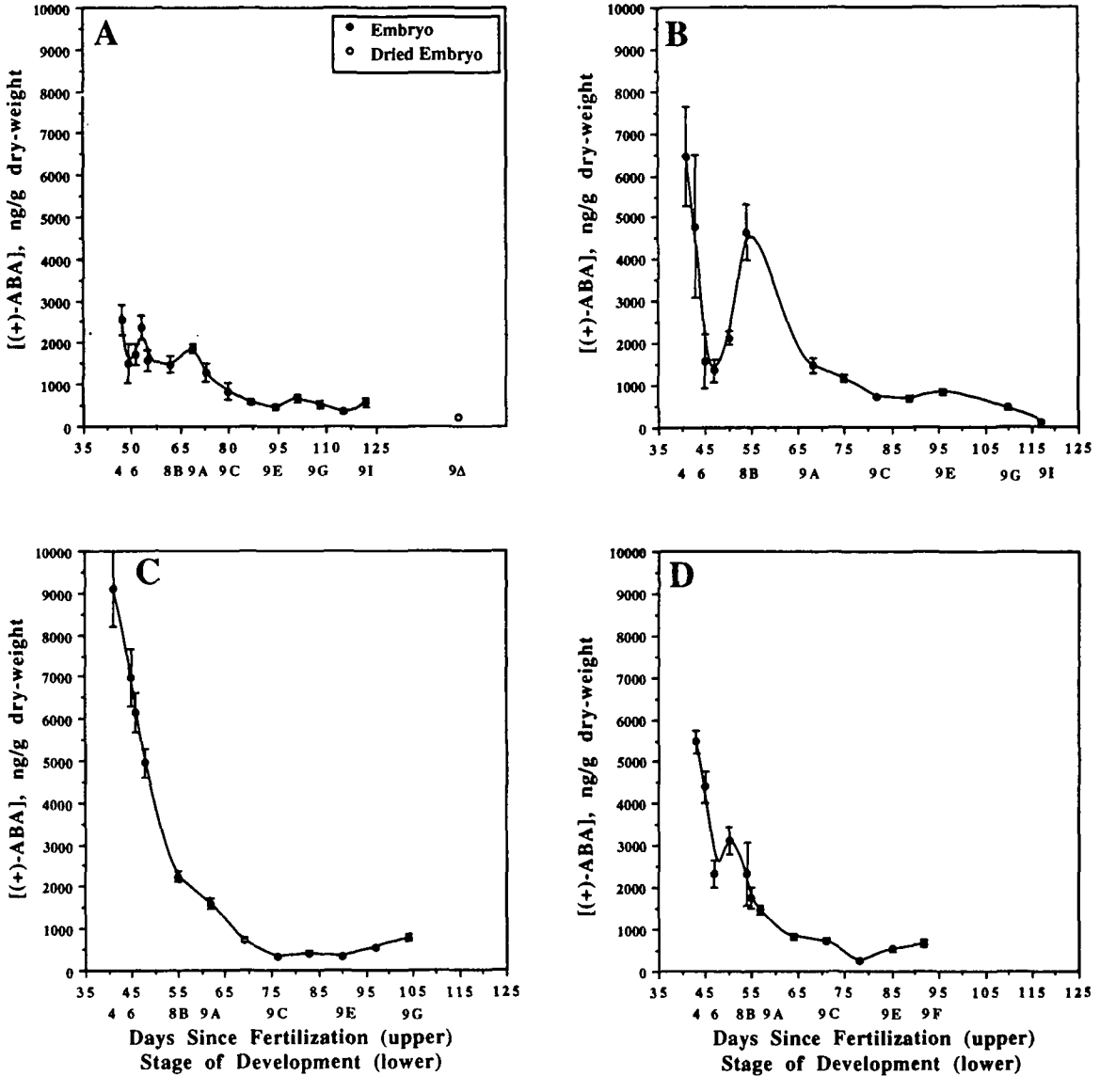

Figure 30. Comparison of (+)-ABA concentrations in **1992 zygotic embryos on a g dry-weight basis**. **(A)** UC_{92}. **(B)** WV_{92}. **(C)** WA_{92}. **(D)** WB_{92}. 9Δ denotes fully-dried seed. Error bars indicate the 95% confidence intervals.

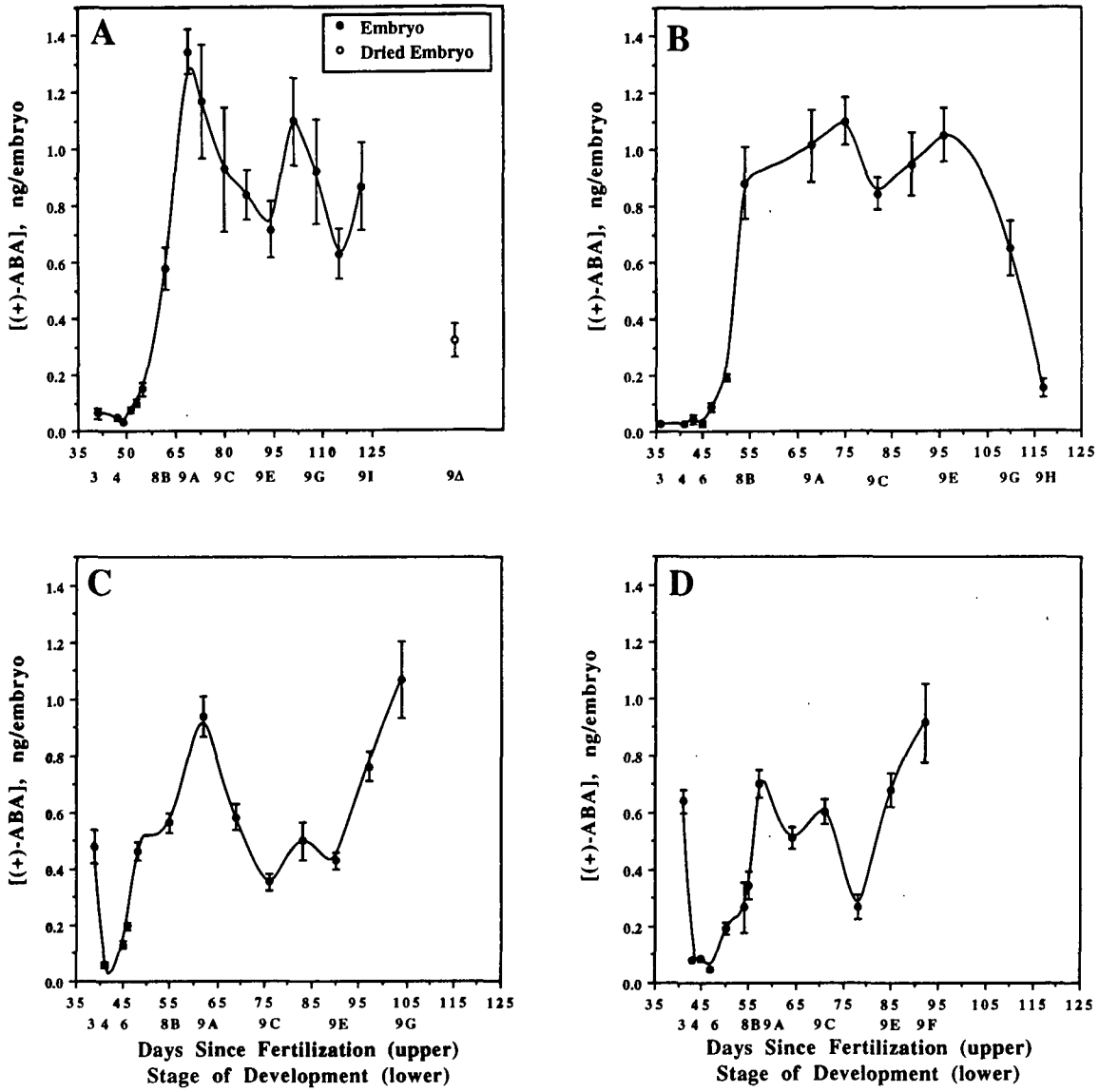

Figure 31. Comparison of (+)-ABA contents in **1992 zygotic embryos on a per organ basis.** **(A)** UC_{92}. **(B)** WV_{92}. **(C)** WA_{92}. **(D)** WB_{92}. 9Δ denotes fully-dried seed. Error bars indicate the 95% confidence intervals.

On a per embryo basis, all four mother trees displayed the same general trend: very low ABA contents early in development, followed by a rapid rise at mid development (Stage 7), peaking at about Stage 9A before remaining constant for UC_{92}, WA_{92}, and WB_{92}, and dropping to almost its original level for WV_{92} at cone maturity. For UC_{92}, ABA dropped rapidly from cone (9I) to seed maturity (9Δ), i.e., dormant seed. A second smaller peak occurred in each tree at mid-to-late Stage 9. The WA_{92} and WB_{92} trees were grown in the same locale (Lyons, GA) and displayed remarkable similarity in peaks, as well as in the ABA increase that occurred late in development, but was not seen in the UC_{92} and WV_{92} samples. The high level of ABA at Stage 3 for WA_{92} and WB_{92} was most likely due to errors in estimating the number of embryos collected, which in one case was less than 4 embryos.

1992 Trends - Megagametophyte

Changes in endogenous ABA levels found in developing megagametophytes are illustrated on a dry-weight basis (Fig. 32) and on a per embryo basis (Fig. 33) for seeds taken from the four mother trees (WV_{92}, UC_{92}, WA_{92}, and WB_{92}) and representing Stage 3 to cone maturity (seed maturity for UC_{92}). Similar to the trends found in embryos, endogenous (+)-ABA concentrations were highest during the early stages of development and dropped to their lowest concentrations at cone and seed (UC_{92}) maturity. Peaks in megagametophyte ABA concentration occurred at stages of development different from those found in the embryos: Stage 7 for UC_{92}; Stages 7, 9A, and 9G for WV_{92}; Stages 5 and 9A for WA_{92}; and Stages 8A and 9A for WB_{92}. Megagametophytic ABA concentrations at Stage 3 (1,200-2,300 ng/g dry-weight) were similar for the four trees, as were the concentrations during late development.

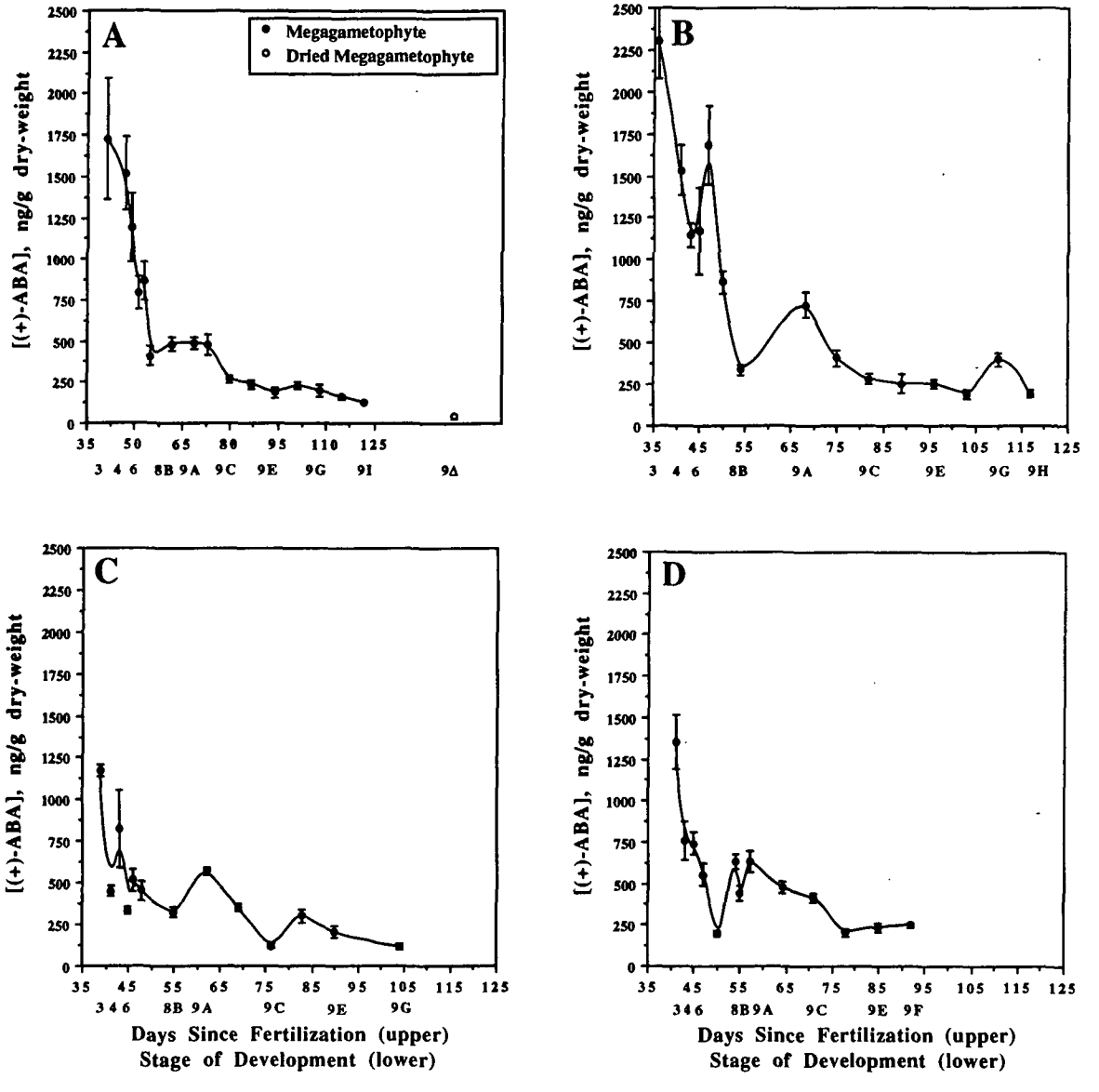

Figure 32. Comparison of (+)-ABA concentrations in **1992 zygotic megagametophytes on a g dry-weight basis**. **(A)** UC_{92}. **(B)** WV_{92}. **(C)** WA_{92}. **(D)** WB_{92}. 9Δ denotes fully dried seed. Error bars indicate the 95% confidence intervals.

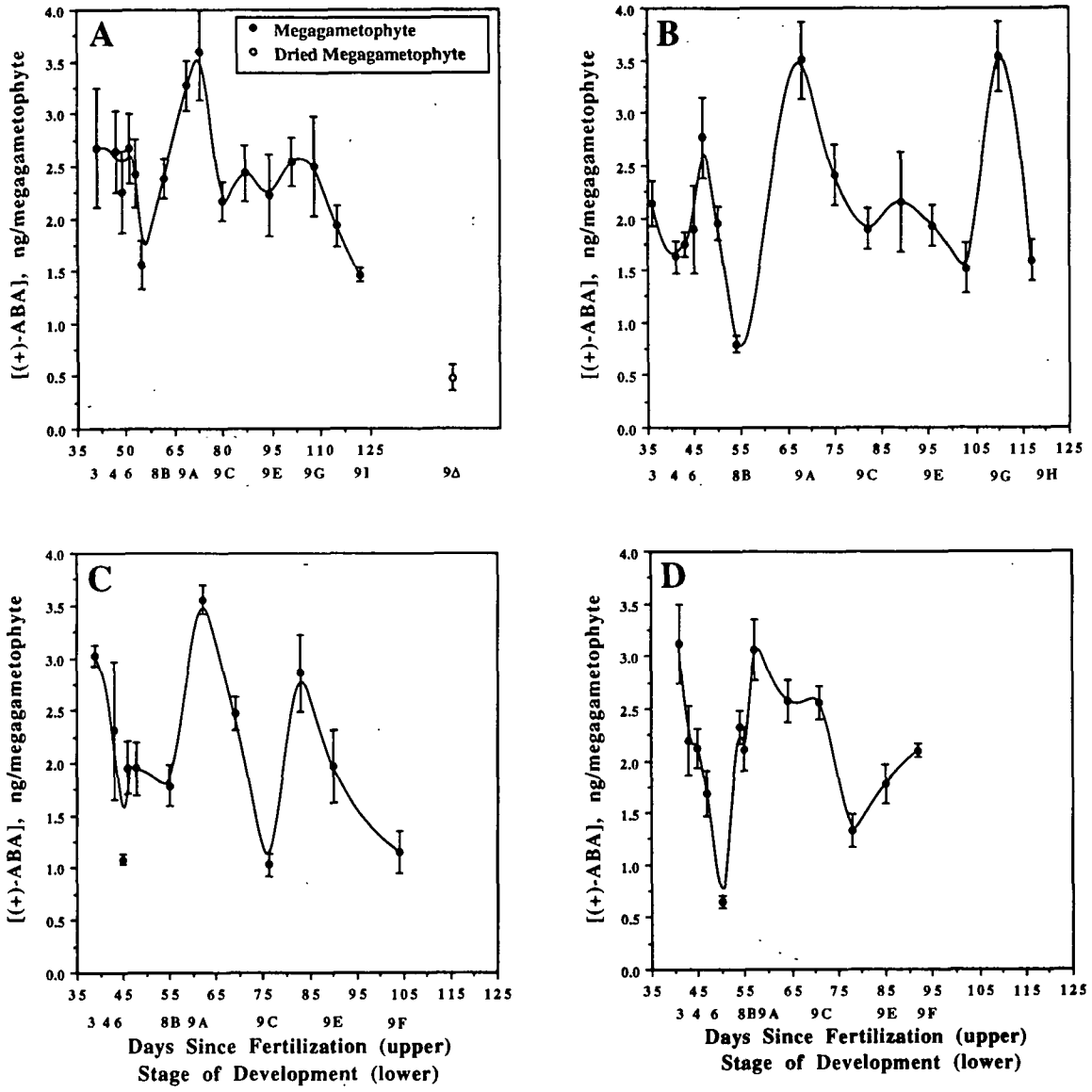

Figure 33. Comparison of (+)-ABA contents in **1992 zygotic megagametophytes on a per organ basis**. **(A)** UC_{92}. **(B)** WV_{92}. **(C)** WA_{92}. **(D)** WB_{92}. 9Δ denotes fully dried seed. Error bars indicate the 95% confidence intervals.

In comparing embryo (Fig. 30) to megagametophyte (Fig. 32) results on a dry-weight basis, embryonic ABA was always much higher than megagametophytic ABA, and the peaks between the two tissues rarely coincided with each other.

On a per megagametophyte basis, there were two similarities between the four mother trees. One trend in common was a large drop in ABA content around Stages 7-8B, just prior to a substantial increase that occurred with the onset of Stage 9. The other similarity appeared to be a second increase late in development, at 9F and 9G for UC_{92}; 9F for WV_{92} and WB_{92}; and 9D for WA_{92}. For UC_{92}, megagametophytic ABA continued to drop to seed maturity. Comparing the embryo (Fig. 31) to megagametophyte (Fig. 33) results on a per organ basis, megagametophytic ABA was always much higher than embryonic ABA, except at seed maturity (UC_{92}). Also, the major embryonic ABA peaks appeared to coincide with the first drop in megagametophytic ABA, suggesting a causal relationship. The decrease in megagametophytic ABA on a dry-weight basis appeared to result primarily from the increasing mass of the tissue since the same data expressed on a organ basis was relatively constant over the time range.

1992 Trends - Suspensor

Changes in endogenous ABA levels found in developing suspensors are illustrated on a dry-weight basis (Fig. 34A) and on a per suspensor basis (Fig. 34B) for tissues from Stage 3 (DSF 36) to mid-Stage 9 (DSF 72) taken from the four mother trees (WV_{92}, UC_{92}, WA_{92}, and WB_{92}). After early-to-mid Stage 9 (DSF 72+), the suspensors had degenerated and so were not analyzed. In the course of this work, the extraction solvent also became

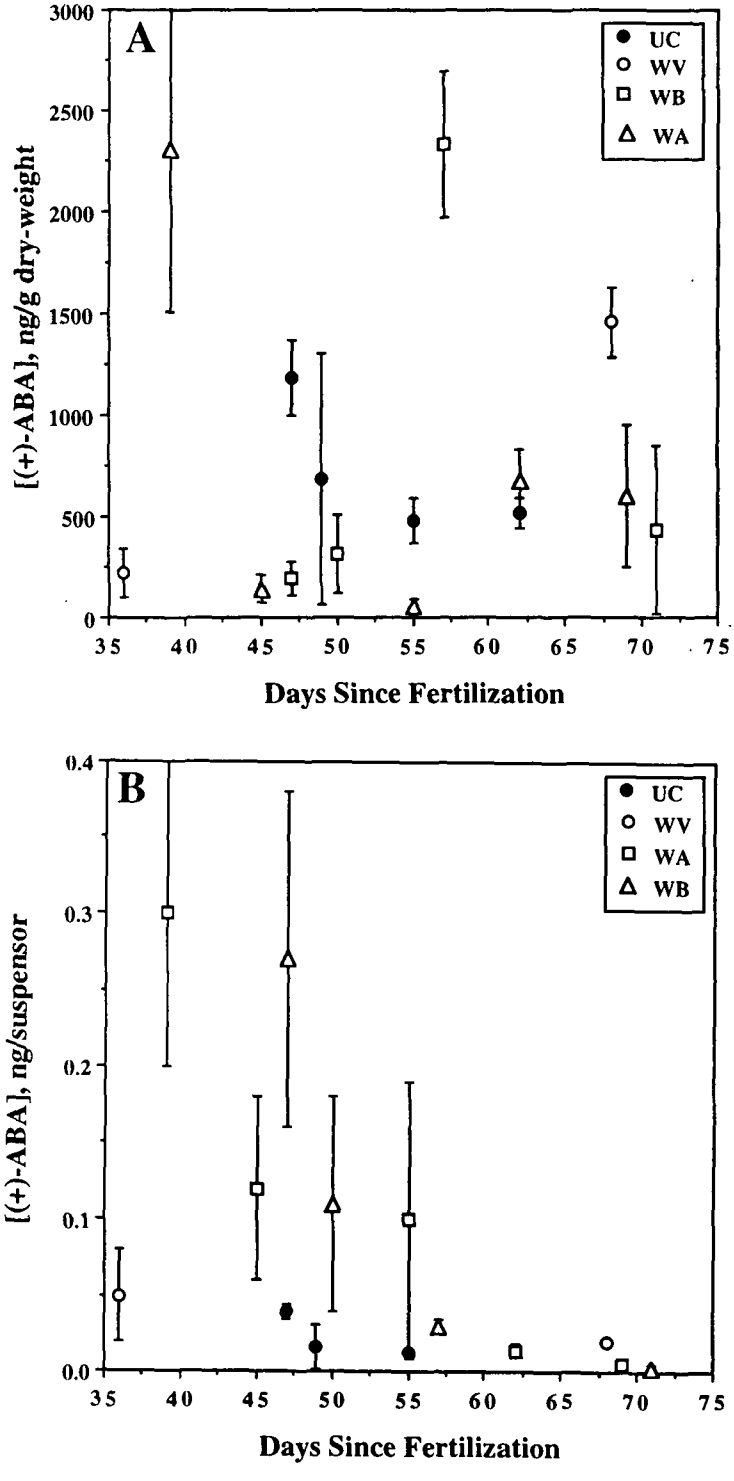

Figure 34. Comparison of (+)-ABA levels in **1992 zygotic suspensors** from UC_{92}, WV_{92}, WA_{92}, and WB_{92}. **(A)** Dry-weight basis. **(B)** Suspensor basis. Error bars indicate the 95% confidence intervals.

contaminated with ABA (presumably via a contaminated pipet tip) and as a result, 12 samples from all the mother trees were lost. Also, difficulty in weighing the minute amounts of tissue caused high variability in the results, as illustrated by the wide 95% confidence intervals. On a dry-weight basis, there did not appear to be any similarities between the trees or any general trends. ABA appeared to be relatively low (~500 ng/g dry-weight) in comparison to embryonic and megagametophytic ABA. On a per suspensor basis, there appeared to be higher levels of ABA early in development decreasing to low or undetectable levels late in development, a trend that was opposite that for embryonic ABA. On a per organ basis, ABA content in the suspensors appeared greater than embryonic ABA early in development.

1993 Trends - Whole Ovule, Embryo, Embryo + Suspensor, and Megagametophyte

Changes in the concentrations of endogenous ABA in whole ovules (embryo, suspensor, and megagametophyte), as well as in the individual seed parts (whole ovule, embryo, embryo + suspensor, and megagametophyte) from seven days prior to fertilization to seed maturity on a dry-weight basis are illustrated for WA_{93} in Fig. 35A and 35B, respectively. ABA levels calculated on a fresh-weight basis and on a per organ basis are shown in Fig. 36A and 36B, respectively. ABA levels were also calculated on a μM basis assuming the endogenous ABA was uniformly distributed throughout the available water (Fig. 37).

Figure 35A, constructed by adding the ABA concentrations from the individual seed parts, endogenous ABA concentrations in the entire seed showed two maxima: one occurring immediately after fertilization and the second occurring at Stage 7. Fig. 35B shows which tissues were contributing to the peak ABA values: megagametophytic tissues for the

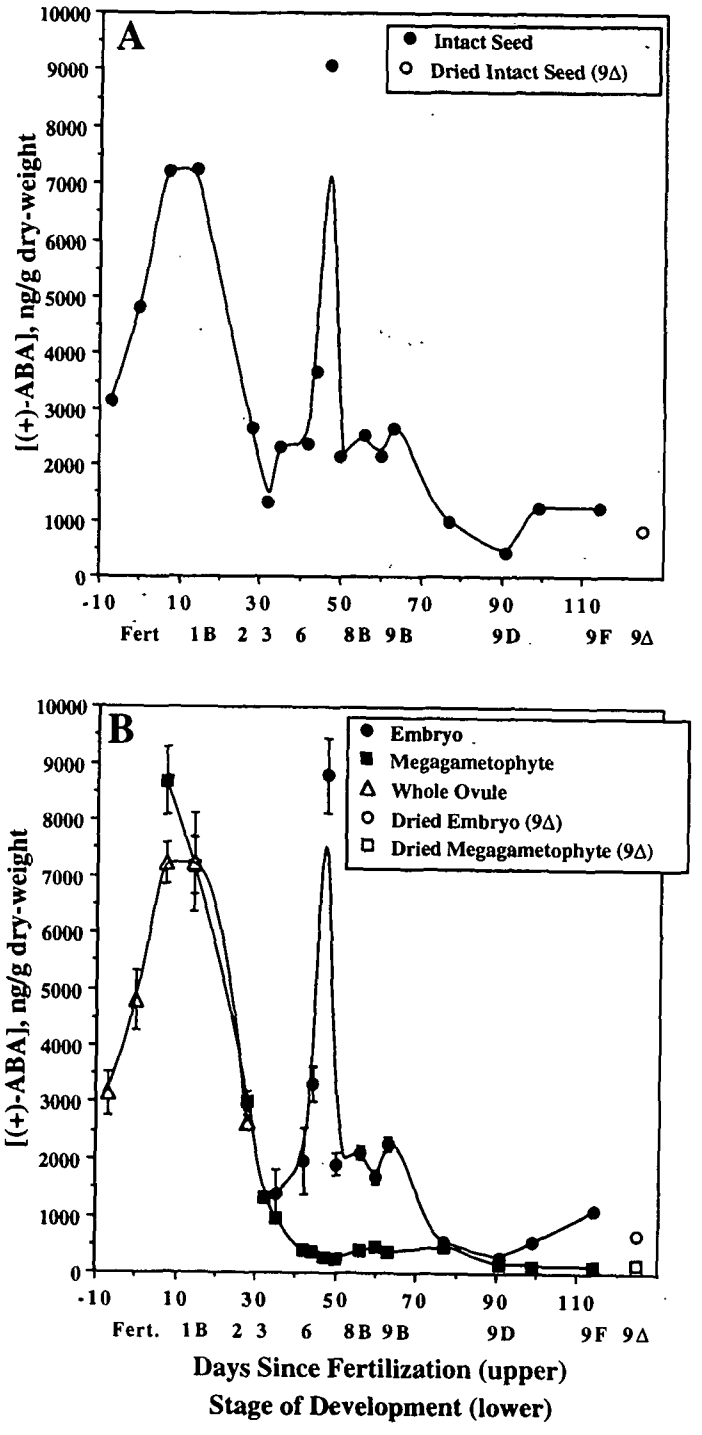

Figure 35. (+)-ABA concentrations in **WA_{93} zygotic whole ovules, embryos, and megagametophytes on a g dry-weight basis.** **(A)** Intact seed. **(B)** Individual seed parts. Embryo + suspensor tissues were collected during Stages 1A to 2 (not shown) remained constant at approximately 1,000 ng. Error bars indicate the 95% confidence intervals.

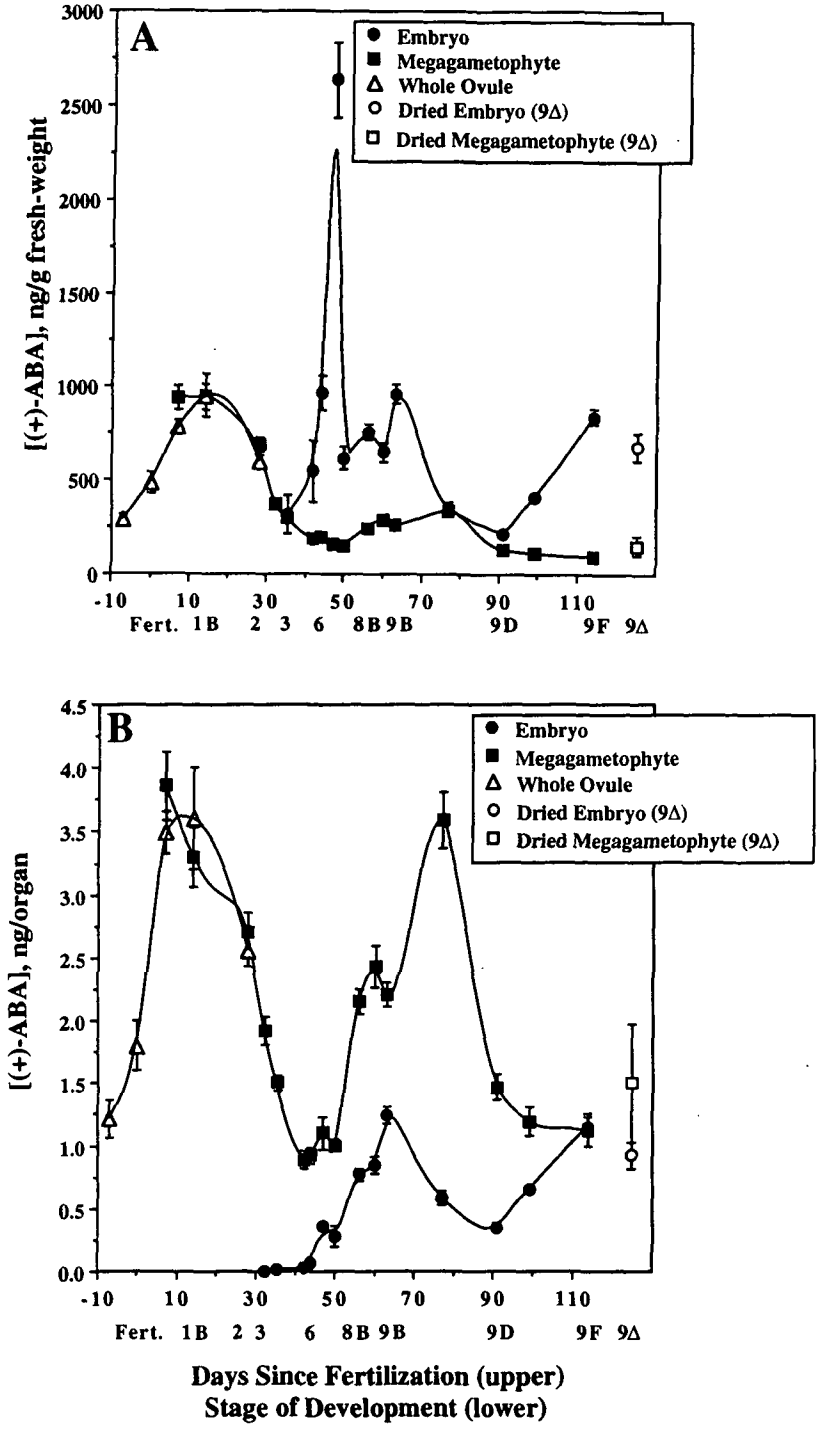

Figure 36. (+)-ABA levels in **WA_{93} zygotic whole ovules, embryos, and mega-gametophytes on a (A) g fresh-weight basis and (B) per organ basis.** Embryo + suspensor tissues were collected during Stages 1A to 2 (not shown) remained constant at approximately 250 ng/g fresh-weight and 0.01 ng/organ. Error bars indicate the 95% confidence intervals.

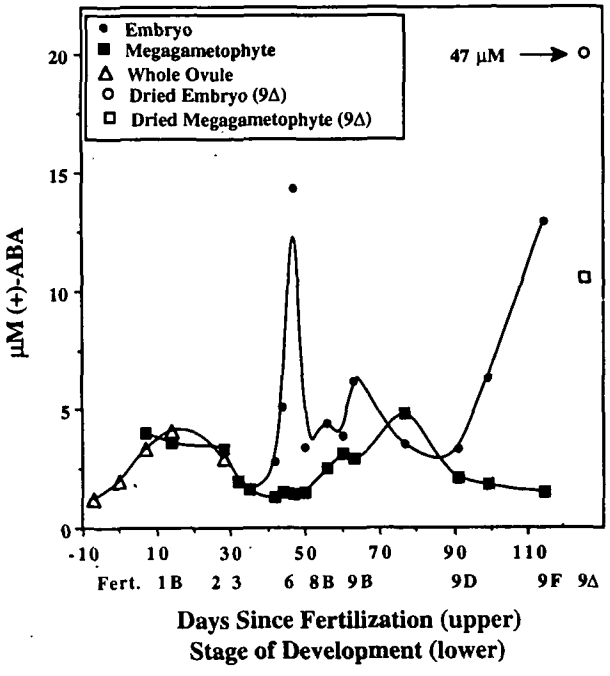

Figure 37. (+)-ABA concentrations in **zygotic whole ovules, embryos, and megagametophytes on a per µM basis** from WA_{93}.

first peak and embryonic tissues for the second peak. The whole ovule ABA concentration was relatively high one week prior to fertilization (3,200 ng/g dry-weight), peaked during Stages 1A and 1B (7,200 ng/g dry-weight), then declined at Stage 2. ABA from embryos + suspensors (not included on the graph) from Stages 1A to 2 remain fairly constant at approximately 1,000 ng/g dry-weight. Megagametophytic ABA was very high at Stage 1A (8,700 ng/g dry-weight) and rapidly declined until Stage 5, where it leveled off (400 ng/g dry-weight), before slowly dropped until seed maturity (150 ng/g dry-weight). Embryonic ABA increased very rapidly from Stage 4 (1,400 ng/g dry-weight) to 7 (9,000 ng/g dry-weight), then rapidly declined to Stage 8A (2,000 ng/g dry-weight), maintained a shoulder peak from Stages 8B to 9B, then declined to Stage 9D before increasing slowly to cone maturity (9F) and dropping at seed maturity (9Δ). The maximum embryonic ABA level at Stage 7 was

approximately equal to the megagametophytic ABA level at Stage 1A.

The trends for the tissues on a fresh-weight basis (Fig. 36A) were similar to those found on a dry-weight basis, with the exception that the embryonic ABA concentration was much greater than the whole ovule or megagametophytic ABA concentrations. Embryonic ABA showed a steady increase to cone maturity, but then decreased slightly at seed maturity.

On a per organ basis (Fig. 36B), whole ovule ABA increased rapidly from one week prior to fertilization (1.2 ng) to Stage 1B (3.5 ng), and then began to decrease to Stage 2 (2.6 ng). Embryo + suspensor tissue ABA remained extremely low, but detectable (0.01 ng), from Stage 1A to 2. Megagametophytic ABA was very high, similar to that in whole ovules at Stages 1A and 1B, but then decreased, again following the whole ovule trend, until Stage 5. ABA then remained constant (0.9 ng) until Stage 8A, peaked slightly at Stage 9A (2.4 ng), and then increased rapidly to Stage 9C (3.6 ng). After this second large peak (9C), megagametophytic ABA again decreased to cone maturity (1.1 ng) before increasing slightly at seed maturity (1.5 ng). Embryonic ABA was extremely low from Stage 3-5 (0.01 ng), increased from Stage 6 (0.07 ng) to 7 (0.4 ng), and then dropped slightly at Stage 8A (0.3 ng) before rapidly increasing to Stage 9A (1.3 ng). ABA then slowly declined until Stage 9D (0.65 ng) when it began to rise slowly to cone maturity (1.5 ng) before it dropped again to seed maturity (0.92 ng). Although there was a slight lag, the first drop in megagametophytic ABA preceded an increase in embryonic ABA content suggesting a causal relationship between the two tissues. Afterwards, ABA content in both tissues increased concurrently until embryonic ABA peaked and dropped corresponding to a small peak in megagametophyt-

ic ABA. Then, as development continued, the embryonic and megagametophytic ABA contents fluctuated out of sequence.

On a μM basis, whole ovule ABA increased rapidly from one week prior to fertilization (1.2 μM) to Stage 1B (4.1 μM) and then began to decrease to Stage 2 (2.9 μM). Embryo + suspensor tissue ABA remained low from Stage 1A to 2 (0.5-1.0 μM). Megagametophytic ABA was similar to that in whole ovules from Stages 1A to 2, but then decreased until Stage 5. ABA then remained constant (1.5 μM) until Stage 8B, and then increased rapidly to Stage 9C (4.8 μM). After this peak, megagametophytic ABA decreased to cone maturity (1.5 μM). Dried seed (9Δ) contained a very high concentration of megagametophytic ABA (10.5 μM). Embryonic ABA increased rapidly from Stage 4 (1.6 μM) to Stage 7 (14.3 μM), and then dropped rapidly to Stage 8A (3.4 μM). Embryonic ABA went on to peak at Stage 9B (6.2 μM) and again at cone maturity (12.9 μM). Embryonic ABA in dried seed was extremely high (47 μM).

Comparison of 1992 and 1993 Trends

Figure 38 compares ABA trends for tissues from the WA tree during the 1992 and 1993 growing season for (A) embryos and (B) megagametophytes on a dry-weight basis. The trends were very similar for both tissues. On a DSF basis, the embryonic ABA trends (Fig. 38A) were almost identical; peak ABA values were only five days apart (assuming that Stage 4 for 1992 was the peak value). The ABA concentration in both years dropped very rapidly after peaking, a shoulder appeared in the 1993 trend, then both slowly increased until cone maturity. In 1993, ABA concentrations dropped slightly from cone maturity (1,106 ng) to

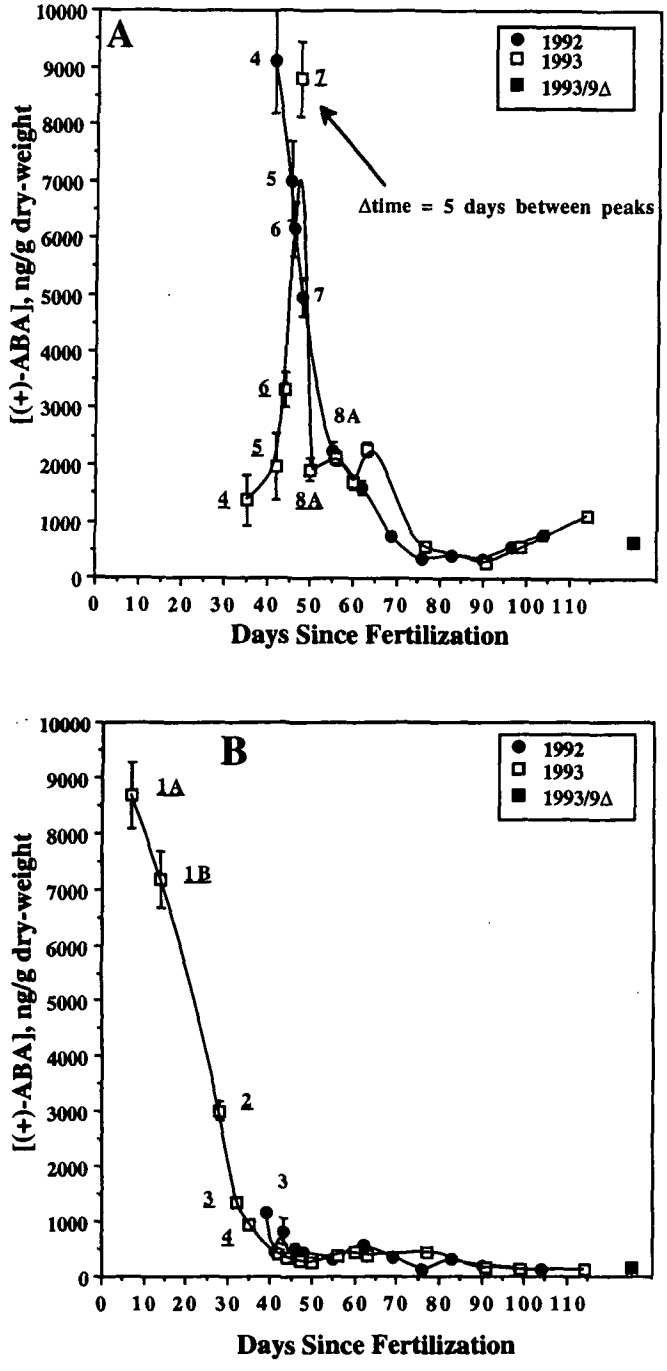

Figure 38. Comparison of (+)-ABA concentrations from **1992 and 1993 on a g dry-weight basis** from WA. **(A)** Embryo. **(B)** Megagametophyte. Numbers on graphs indicate stage of development. Underline = 1993. Normal = 1992. Error bars indicate the 95% confidence intervals.

seed maturity (673 ng). The trends on a SOD basis were not similar. In 1992, Stages 4-6 were relatively low in ABA, whereas in 1993, the values were very high. After Stage 8A, ABA values were similar. These results would suggest that ABA trends were less a function of SOD than of DSF.

The trends for 1992 and 1993 megagametophytic ABA (Fig. 38B) were also very similar on a DSF and SOD basis, again to the extent of tissues collected during 1992. ABA concentrations decreased from approximately 1,200 ng/g dry-weight at Stage 3 to about 115 ng/g dry-weight at cone maturity during 1993.

Figure 39 compares ABA trends for WA during 1992 and 1993 for (A) embryos and (B) megagametophytes on a per organ basis. The trends for embryos (Fig. 39A) showed identical changes in ABA during embryogenesis on a DSF basis, except for the high value of ABA at Stage 3 during 1992. This was very possibly due to an error in counting the smaller than pin head-sized embryos. Although the SOD dates were slightly different between the two years, there were strong similarities in ABA values on a SOD basis. As Fig. 39B illustrates, there were no similarities in megagametophytic ABA from 1992 and 1993. In fact, the highest and lowest ABA values occurred at the same stage in different years. Seed maturity appeared to occur when embryonic ABA was increasing and megagametophytic ABA was at its lowest concentration (100+ DSF). This was found to be true in every case during the 1992 growth season (Figs. 31 and 33).

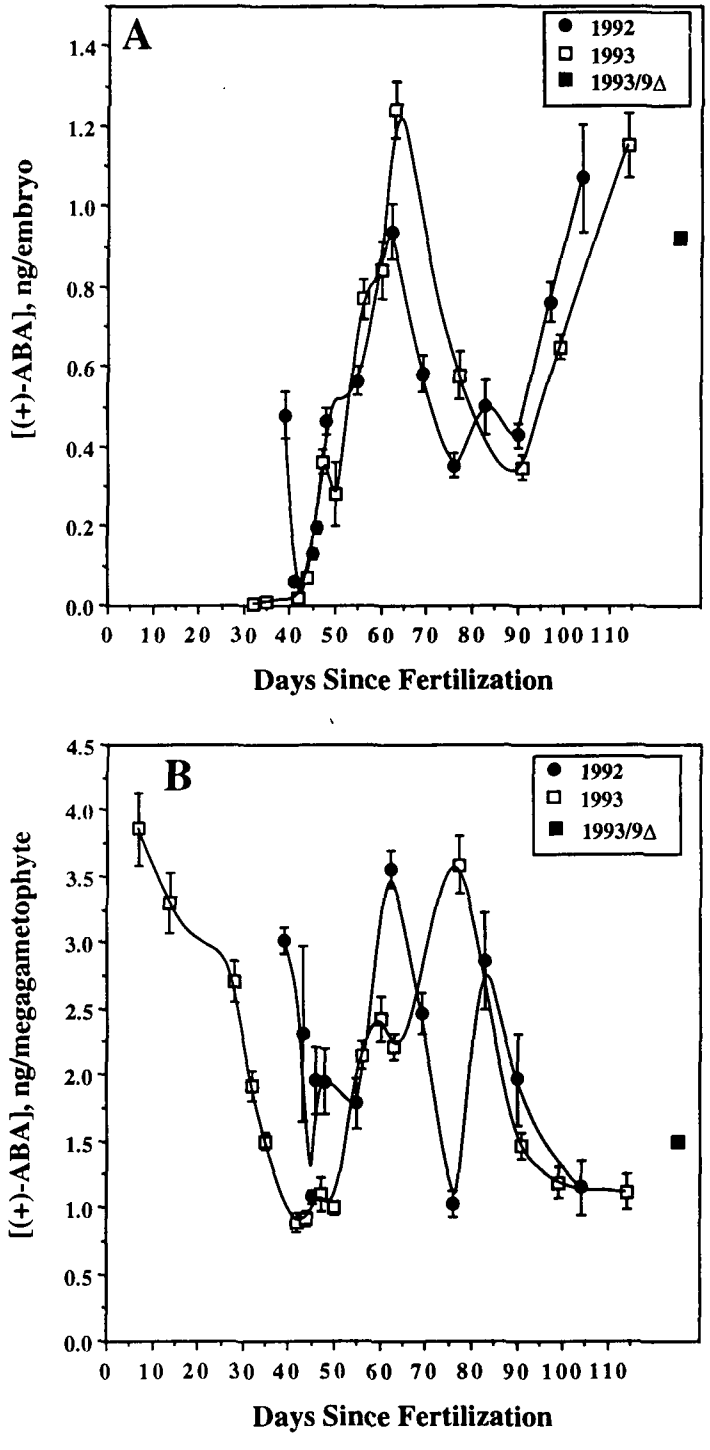

Figure 39. Comparison of (+)-ABA content from **1992 and 1993 on a per organ basis** from WA. **(A)** Embryo. **(B)** Megagametophyte. Error bars indicate the 95% confidence intervals.

Changes in ABA Content Versus Dry-Weight Accumulation

Figure 40A-D illustrates the relationship between ABA on a per embryo basis and the embryo dry-weight accumulation during zygotic embryogenesis for UC_{92}, WV_{92}, WA_{92}, and WB_{92}, respectively, during 1992. Figure 41 shows the relationship during 1993 for WA_{93}. In all plots, a rapid increase in ABA occurred just prior to the exponential increase in dry weight accumulation in the embryo. After peaking, ABA content dropped rapidly, peaked a second time at mid-Stage 9, before dropping (UC_{92} and WV_{92}) or increasing (WA_{92} and WB_{92}) to cone maturity while dry-weight continued to accumulate. This would suggest a causal relationship between ABA content and dry-weight accumulation in embryos. There were no trends between megagametophytic ABA and dry-weight accumulation (not shown).

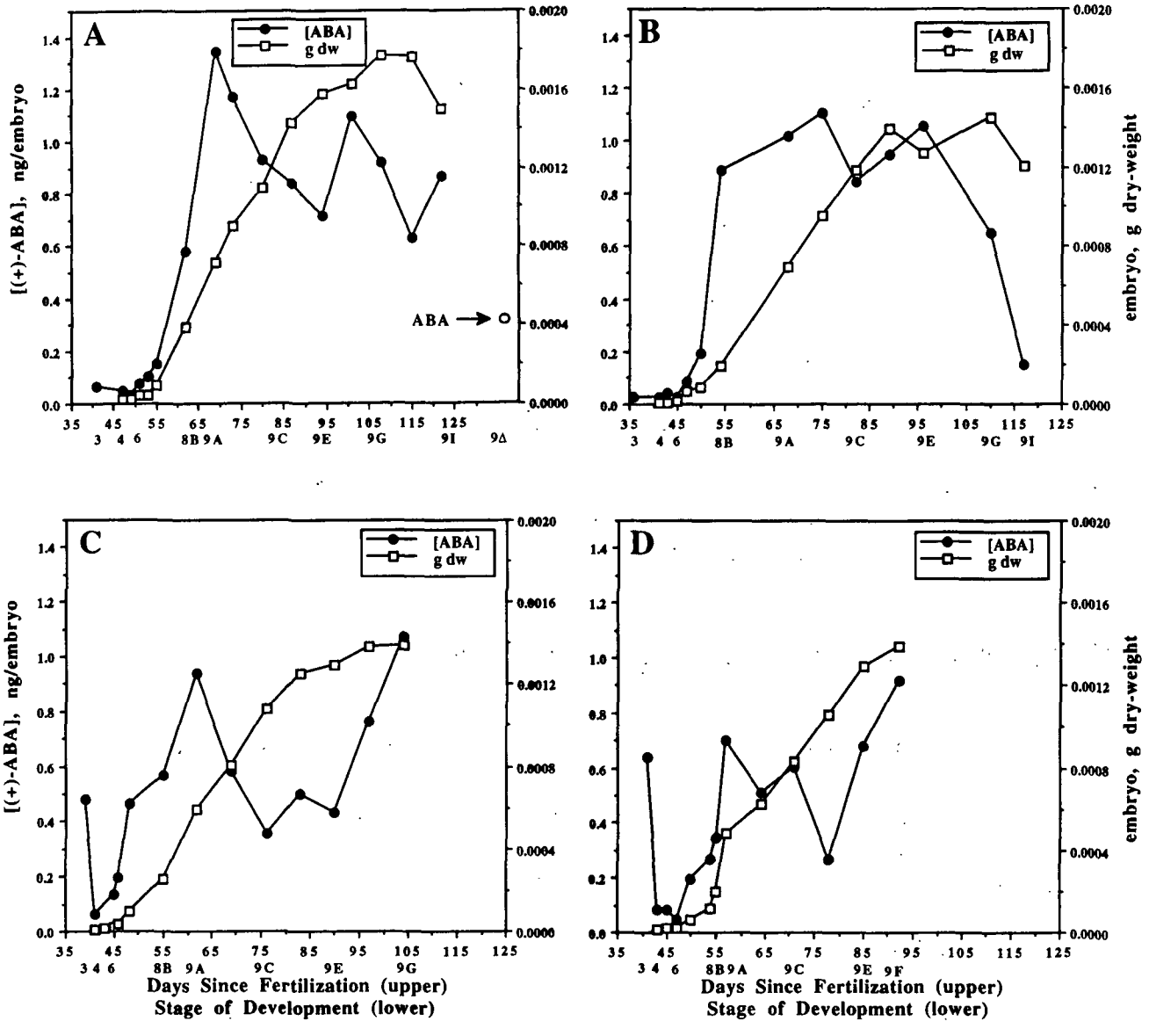

Figure 40. Comparison of (+)-ABA content versus dry-weight accumulation in **1992 zygotic embryos on a per embryo basis.** **(A)** UC_{92}. **(B)** WV_{92}. **(C)** WA_{92}. **(D)** WB_{92}. 9Δ denotes fully dried seed. Error bars indicate the 95% confidence intervals.

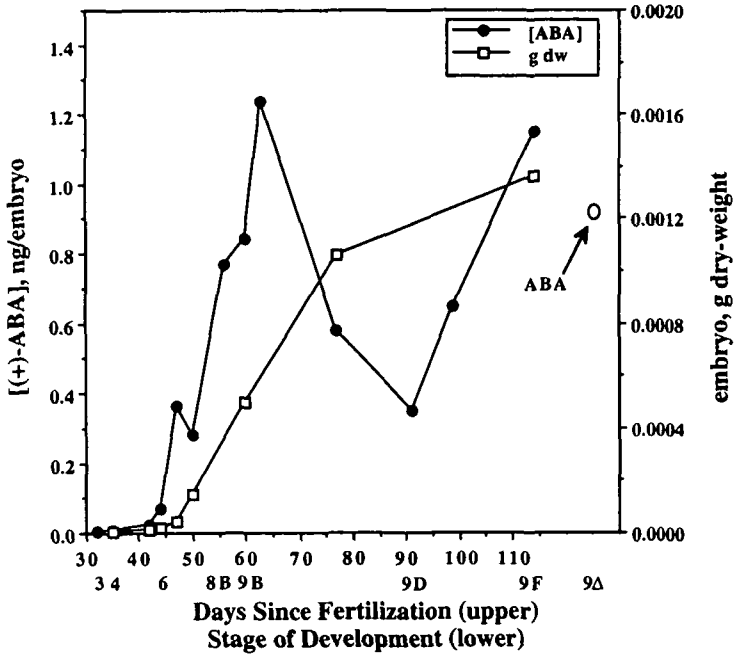

Figure 41. (+)-ABA content versus dry-weight accumulation in **1993 zygotic embryos on a per embryo basis** from WA_{93}. 9Δ denotes fully dried seed. Error bars indicate the 95% confidence intervals.

Changes in ABA Content Versus Desiccation of Tissue

Figure 42 illustrates the relationship for 1993 embryos between endogenous ABA concentrations (g dry-weight) and (A) percent moisture content and (B) mg water content in the embryo. In Fig. 42A, desiccation of the embryo was initiated well before the major peak in ABA, and in Fig. 42B, the ABA peak occurred well before the increase in water content of the embryo. The relationship between endogenous ABA on a fresh-weight and per organ basis versus percent moisture content are similar to Fig. 42A, i.e., desiccation of the embryo started well in advance of the ABA peak. This information would suggest that embryonic ABA does not function in initiating or maintaining desiccation in pine seeds.

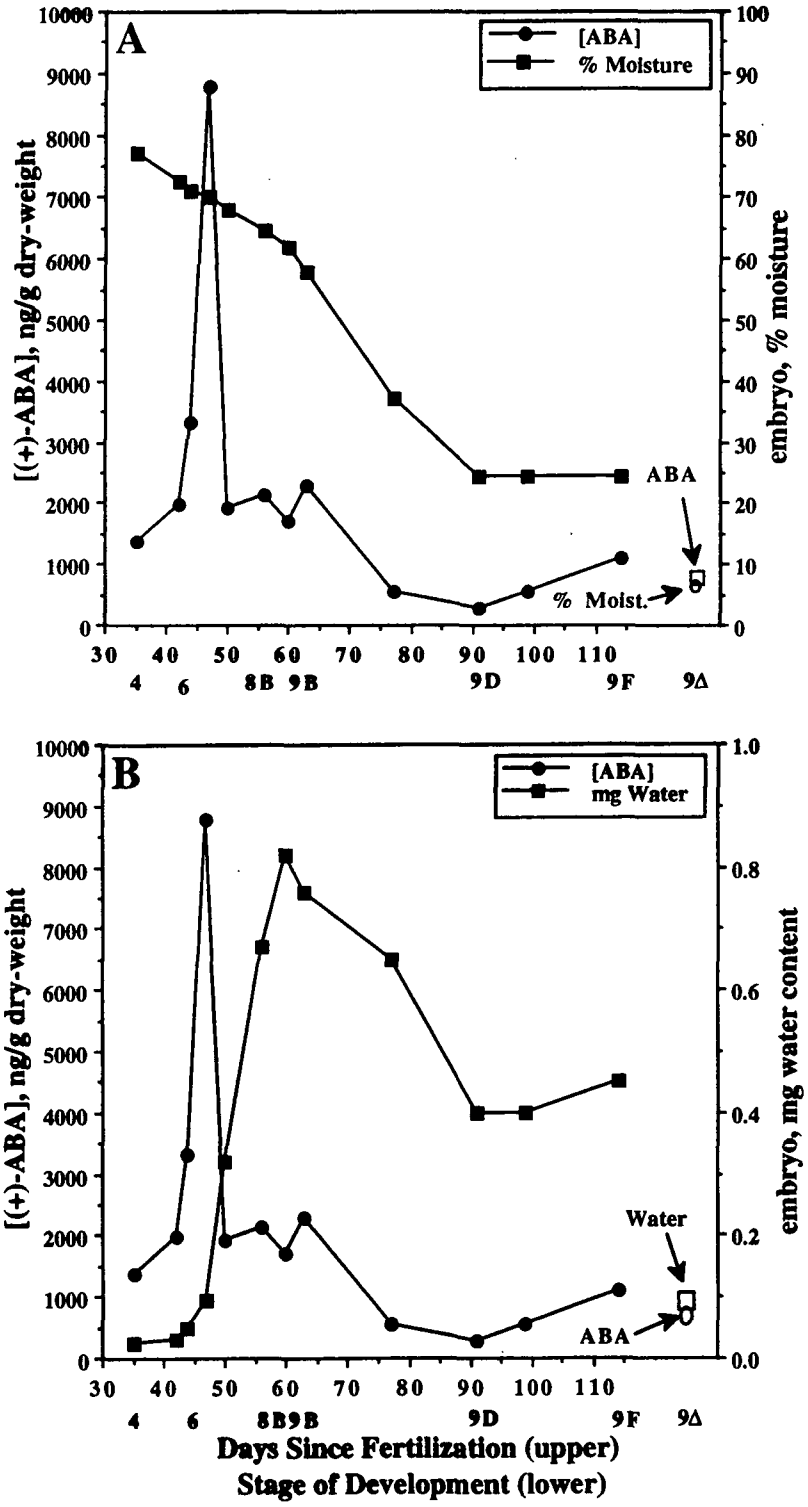

Figure 42. Causal Relationship for WA_{93} **embryonic ABA on a g dry-weight basis** versus (A) Percent moisture content. (B) Water content, mg.

Figure 43A illustrates the change in endogenous ABA concentrations (g dry-weight) versus percent moisture content in 1993 megagametophytes. The drop in ABA occurred concurrently with the drop in percent moisture content suggesting a causal relationship. The water content in the megagametophytes remained relatively constant throughout embryogenesis (Fig. 43B) until forcibly reduced by drying (9Δ). In comparing Fig. 42A and 43A, there may be a causal relationship between megagametophytic ABA and the desiccation of the embryo during development, suggesting that megagametophytic ABA may initiate and control desiccation in both embryo and megagametophytic tissues.

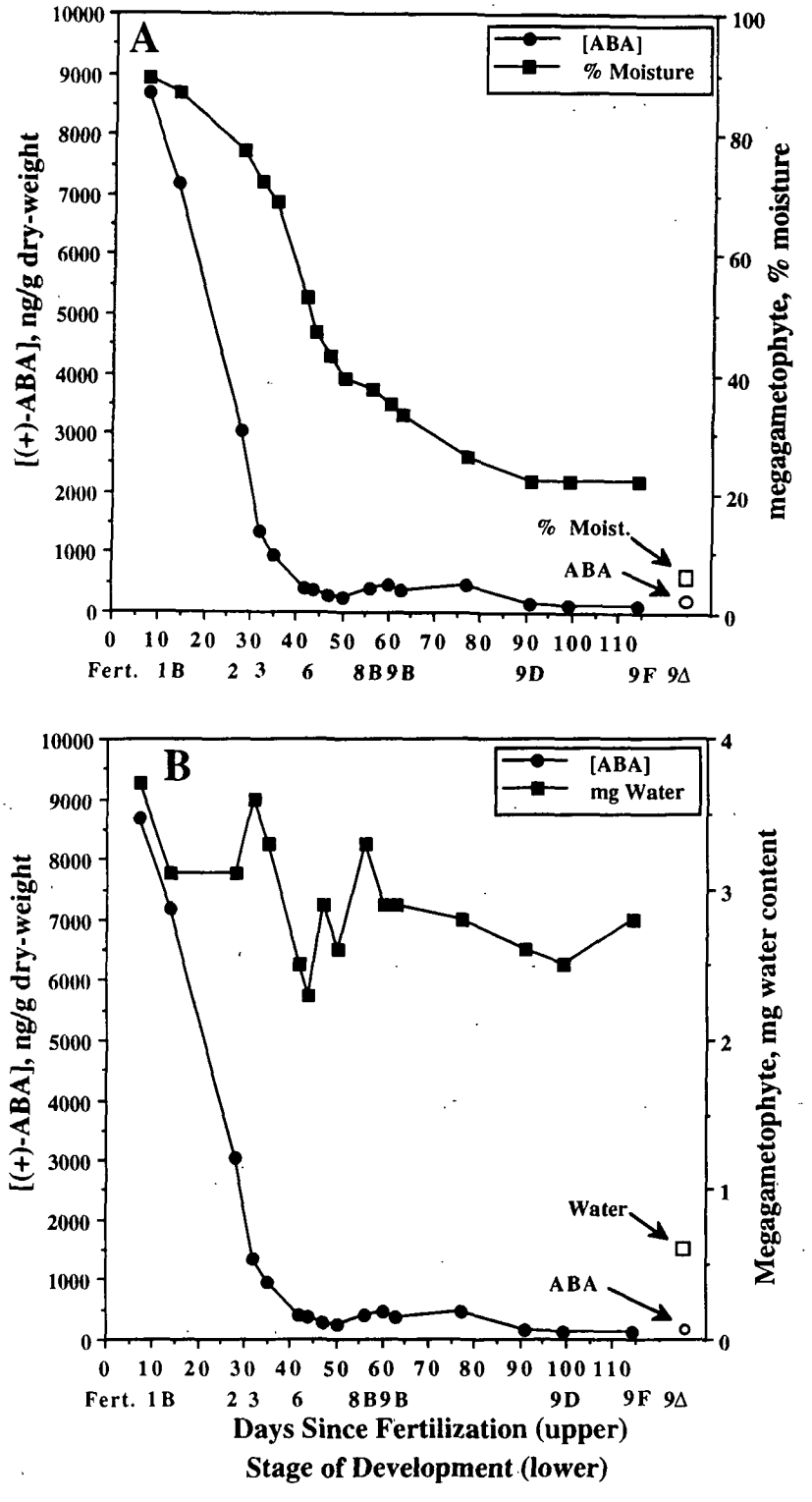

Figure 43. Causal Relationship for **WA_{93} megagametophytic ABA on a g dry-weight basis** versus **(A)** Percent moisture content. **(B)** Water content, mg.

DISCUSSION

EFFECTS OF METHODOLOGY ON ENDOGENOUS ABA CONCENTRATIONS

ABA levels were measured in individual seed parts throughout loblolly pine embryogenesis. These results coupled with studies of specific events in seed development, e.g., transcription of specific storage proteins (113,115,130), should facilitate assessment of the *in-vivo* role of ABA in conifer seed development. Unfortunately, certain of the methods used in the collection of tissue for this study appeared to have altered the absolute levels of endogenous ABA in the fractionated seed tissues.

Effects of Tissue Dissection and Storage Methods

Endogenous ABA concentrations were significantly increased (approximately 50%) by separation of embryo, suspensor, and megagametophyte tissues during dissection, but were apparently unaffected by washing of embryo and suspensor tissues (Table 18). The dissection procedure, i.e., from opening of the seed coat to freezing in liquid nitrogen, was performed as quickly as possible (1.5-2 minutes), under minimal lighting and at RT in an effort to reduce the stress that would inevitably occur during the method. Unfortunately, endogenous ABA concentrations still rose in response to this treatment. It is known that a reduction in turgor pressure or cell volume will act as a trigger to increase ABA biosynthesis. For instance, water-induced stress resulted in a 40-fold increase in ABA in leaf stomata of *Vicia faba* L. within ten minutes (173). However, the location of the trigger in the pathway is unknown (174). Also, in many cases, ABA moves rapidly from tissue to tissue. Could damage-

induced ABA form preferentially in one tissue, then spread to others? For this study, dissections had to be performed since it could not be assumed that ABA was homogeneously distributed throughout physiologically different tissues, i.e., embryo + suspensor or, for that matter, physiologically similar tissues, i.e., embryos. Freezing the tissues in liquid nitrogen prior to dissection proved impractical as the tissues broke apart and were difficult to cleanly separate from each other. This is the first report on the effects of seed dissection on endogenous ABA concentrations in embryonic tissues.

Storage of lyophilized tissues at -80°C, nitrogen/vacuum atmosphere, and in the dark also was not sufficient in maintaining endogenous ABA concentrations (Table 19). Despite very stringent storage conditions, ABA concentrations increased approximately 28% by 18 days after freezing. This increase in ABA may have been the result of stress-induced synthesis or hydrolysis of ABA-conjugates (13,19) during the initial drying. After 106 days, the artificially elevated ABA concentrations had remained constant in comparison to levels at day 18. Thus, analysis of ABA would best be performed on freshly collected tissues, e.g., dropping dissected tissues into methanol extraction solvent maintained at ~-40°C in a dry ice/acetone bath. Unfortunately, the time from tissue collection to ABA analysis varied from 136 to 484 days between the four mother trees during 1992. The time from collection to analysis for each mother tree and tissue type was essentially the same so it is unlikely that the overall ABA trend for a given mother tree would be affected, i.e., appearance of a peak. The large time difference between mother trees did not result in generally higher or lower relative ABA concentrations as tissues that were in storage the longest had greater ABA than tissues with the shortest storage time. This is the first report on the effects of storage on endogenous

ABA concentrations.

Stage Of Development and the Effects of Bulking Tissues versus Days Since Fertilization

The advantages and disadvantages of using SOD as a time scale were discussed in Chapter One, and the SOD method for this work was detailed earlier in this Chapter. The SOD method worked very well in collecting tissues, and had the important advantage of allowing direct comparison of the loblolly pine somatic embryogenesis system with zygotic embryogenesis. A disadvantage to using the described method was found during Stage 9 development, which extended for 6-9 weeks (designated 9A-I on the graphs), because there were no obvious visual changes in morphology. Although the original intent had been to separate these tissues by percent moisture content, this would have resulted in a loss of information. Assigning the Stage 9 embryos to sub-stages A-I retained all the data (necessary since biochemical and physiological changes were still occurring), but resulted in two different scales for the graphs: a developmental scale (1-9), and a time-based scale (9A-I). In retrospect, a single time-based scale might have been preferable since it appears that the ABA trends were more a function of DSF than SOD (Fig. 38 and 39).

Combining samples of tissues from the same developmental stage, or bulking, had the advantage of increasing the amount of tissue available for extraction, which was critical for analysis of low-mass Stage 3-7 tissues. However, this may have obscured changes in ABA that occurred on a calendar basis (DSF). In most cases (see Appendix 1), identical stages from a two week period were bulked, but there were a few instances in which identical stages from a three week period were combined. The disadvantage of bulking is that it would

force ABA to be stage related. If the assumption that ABA was related to morphological changes had been accurate, then bulking tissues over a two to three week period would have been an acceptable practice. If ABA was not related to morphological change, but rather to linear time, then the absolute concentration obtained at each stage would be imprecise, thereby adding to the variability of the result. Variability tended to be highest in bulked tissues, but these same tissues were also difficult to weigh accurately, despite the bulking, and this, too, could have added to the imprecision. However, the increased variability does suggest that ABA fluctuations during embryogenesis may be more a function of time than changes in morphology. Unfortunately, the only previous references to bulking were by Hsu (75) and Perata et al. (88) who collected beans at each growth stage at different times over the growing season (species had multiple growing cycles over the growing season). They concluded by saying that this procedure validated the ABA fluctuations as possible interference by insufficient sampling was minimized. In the case of bean, this suggested that ABA was related to stage of development, and not linear time. The evidence presented here suggests that this matter is still equivocal.

ENDOGENOUS ABA LEVELS IN LOBLOLLY PINE TISSUES

ABA Trends, Levels, and Distribution

Levels of ABA found in seed parts have been reported on the basis of mass of ABA per unit of tissue weight (dry- and fresh-weight) or per unit water weight, as well as on the basis of ABA per single "organ." Expressing ABA levels by any of these methods falsely assumes that ABA is evenly distributed throughout heterogenous tissues, cell types, or

available water. Despite this failing, each method can sometimes be used to detect the relationship of ABA content to developmental patterns in individual seed parts during embryogenesis (67). Unfortunately, attempts to obtain increasingly homogenous tissue using conventional dissecting techniques brings with them greater potential for upsetting endogenous ABA levels (Table 18). In this work, ABA levels were determined on a dry-weight basis, while fresh-weight, per organ, and μM bases were calculated using tissue moisture contents and weight per organ.

ABA Trends

The principal findings of these investigations of ABA trends during loblolly pine embryogenesis and development were:

1) ABA in whole seeds showed two peaks: Stage 1A and Stage 7 based on a dry-weight basis (Fig. 35A),
2) the whole seed ABA peak at Stage 1A was attributable to ABA in the megagametophyte while the peak at Stage 7 was attributable to the ABA in the embryo (Fig. 35B),
3) in embryos (dry-weight basis), the major ABA peak occurred prior to collection (Stage 4/approximately 43 DSF) during 1992 and at Stage 7 (approximately 47 DSF) during 1993 (Fig. 30 and 35B). This may have been caused by variation in environmental factors from year-to-year, e.g., moisture and temperature,
4) in embryos (organ basis), the major ABA peak occurred at about Stage 9A-

B (57-75 DSF) during both years (Fig. 31 and 38),

5) in megagametophytes (dry-weight basis), the major ABA peak occurred prior to collection (Stage 3/approximately 39 DSF) during 1992 and at Stage 1A (7 DSF) during 1993 (Fig. 32 and 35B),

6) in megagametophytes (organ basis), the overall trend was complex showing a series of rises and falls at different SODs and DSF but, in general, showed two to three peaks (Fig. 33 and 38). These trends in the mega-gametophyte likely reflect system changes in the mother tree,

7) in general, the ABA trends for the four mother trees during 1992 and for the WA tree between 1992 and 1993 were very similar on a dry-weight and organ basis, with the exception of the highly variable megagametophytic ABA, as determined on an organ basis, and

8) in general, ABA levels decreased from cone maturity to seed maturity on a dry-weight and organ basis. ABA levels increased from cone maturity to seed maturity on a μM basis.

This is the first report on trends in ABA levels during conifer embryogenesis and development. These trends were compared to those in well-studied agricultural species. A biphasic pattern (two peaks), such as that found in loblolly pine seed (Fig. 35A), has been noted in several studies of ABA trends in other whole seeds, including wheat grains (33,62, 64), *Triticale* kernels (71), and seeds of pea (20,74) bean (75), tomato (80), *Arabidopsis thaliana* (81), pear (84), and apple (89).

The ABA content in seeds at any moment in time is the net result of its import, synthesis, export, and metabolism, and it is not known which, if any, of these is the major determinant of measured ABA content (45,67,74,175). In whole loblolly pine seeds, the first ABA peak was ascribed to the megagametophyte and the second peak to the embryo (Fig. 30B). Groot et al. (80) determined that for tomato seeds the genotype of the mother plant regulated the ABA content of the testa, which was responsible for the first peak, and the genotype of the embryo and endosperm was accountable for the second peak. Similarly, Karssen et al. (81) showed that the first ABA peak in *Triticale* kernels was regulated by the genome of the mother plant (maternal ABA) while the second ABA peak was controlled by the genome of the embryo (embryonic ABA). Earlier work by Wang et al. (74) and Hsu (75) using pea and bean seeds, respectively, found that an initial ABA peak came from both the testa and embryo, and a second peak came from the embryo alone. Thus, there appears to be a dual origin for ABA found in seeds. If a similar pattern holds for loblolly pine, maternal ABA may be transported into the seed and stored in the megagametophyte early in development, resulting in the peak detected in Stage 1A. If maternal ABA is transported into the seeds of loblolly pine, it is likely that the trigger for this transport is located in the seed tissues. One potential trigger may be fertilization itself which may signal to the mother plant that nutrients and water are needed in the seed. As ABA is transported via both phloem and xylem tissues (45,62), ABA may enter into the seed (megagametophyte) along with photoassimilates during early development (45). Could the megagametophyte be acting as a buffer or filter to keep ABA out of the embryo?

In the individual seed parts, the embryonic ABA (dry-weight) trend was similar to that found in most non-coniferous species (46), i.e., relatively low quantities during early development followed by a maximum during middle to late embryogenesis (Stage 7) and finally dropping to a low level during maturation (Fig. 2A and 2B). This pattern was clearly shown for loblolly pine during 1993, but not during 1992 when tissue collection was not conducted prior to Stage 3. Since the 1992 and 1993 data for WA almost overlapped (Fig. 39), it is not inappropriate to assume that the 1992 data peaked and fell prior to Stage 4. The megagametophytic ABA (dry-weight) trends followed similar patterns to those found in endosperms from barley (33) and maize (67), i.e., high levels very early in development followed by a decline at maturity. On the other hand, peach (90) displayed the opposite trend. The significant drop in loblolly pine embryonic ABA concentration (dry-weight) from DSF 41+ was primarily a function of the increase in dry-weight accumulation by the embryo. On a per embryo basis, ABA was actually increasing, suggesting that it was either being synthesized by the embryo or imported from an outside source, i.e., megagametophyte, suspensor (88), seed coat (176) or maternal tissues (45,74,60). The significant drop in megagametophytic ABA concentration (dry-weight) from Stages 2-8B (Fig. 32 and 35) was probably due, not to a simple increase in dry-weight of the tissues, but more likely represented a reduction in *de-novo* synthesis, or increased export or metabolism, as the ABA on a per organ basis also dropped during this phase. The rapid disappearance of ABA at seed maturity (9Δ) could have resulted from cessation of ABA import and/or synthesis coupled with a continued destruction or conjugation of ABA. A similar drop in ABA content was found during forced drying of wheat grains (62).

On a per organ basis, loblolly pine embryonic ABA trends were similar those found in soybean (41), barley (66), maize (67), pea (74), bean (88), peach (90), and Norway maple (91), i.e., very low levels early in development were followed by a very rapid increase in mid-development and sometimes a slight drop at maturity. Embryonic ABA trends in wheat (64), alfalfa (73), and sunflower (82) showed a similar rise and fall pattern. However, loblolly pine megagametophytic ABA trends were not at all similar to those found in barley (66), maize (67), and peach (90) endosperms, i.e., low levels early in development followed by a slow rise to late development, then dropping at maturity.

ABA Levels and Physiological Significance

The most pertinent question for assessing the relevance of changing ABA concentrations is, are the fluctuations capable of influencing, i.e., triggering or maintaining morphological, biochemical, and/or genetic events, or are they simply statistically significant changes and nothing else? ABA concentration ranging from 1 to 100 μM have been shown to have a significant effect on embryo-specific gene expression (54). In wheat embryos, 10 μM ABA blocked the stimulatory action of GA_3 on poly(A) polymerase activity (177), and in barley, 50 μM ABA induced the accumulation or synthesis of an α-amylase inhibitor (54). During imbibition of soybean seeds, 100 μM ABA delayed the disappearance of maturation (*LEA*) proteins, loss of desiccation tolerance, and germination (178). Although there is great disparity in the concentrations of ABA used in current systems for conifer somatic embryogenesis, 0.38-100 μM have been found effective for encouraging maturation of somatic embryos (10).

Embryonic ABA levels generally ranged from approximately 0.3-3.3 μg/g dry-weight (0.2-1.0 μg/g fresh-weight; 0.01-0.92 ng/embryo) during loblolly pine embryogenesis with a peak value of 8.8 μg/g dry-weight (2.6 μg/g fresh-weight; 1.3 ng/embryo) at Stage 7 in 1993 embryos. Megagametophytic ABA levels generally ranged from approximately 0.2-1.3 μg/g dry-weight (0.2-0.7 μg/g fresh-weight; 0.9-2.7 ng/megagametophyte) with a peak value of 8.7 μg/g dry-weight (0.9 μg/g fresh-weight; 3.9 ng/megagametophyte) at Stage 1A (Fig. 35B and 36A and 36B).

In comparison, the range and peak embryonic ABA concentrations found in a majority of other species studied were much lower than were found in loblolly pine (ranged from 0.004-0.75 μg/g dry-weight with peak values as high as 3 μg) (33,41,54,63,64,67,72,82, 90,91). A few species were somewhat higher (range from 1-8 μg/g dry-weight) (69,73,77,94), and in one case (alfalfa), significantly higher (range of 7-33 μg/g dry-weight with a peak value of 33 μg) (73). Only four species had embryonic ABA ranges and peak values similar to loblolly pine: bean (75), cacao (78), runner bean (88), and apple (89). Therefore, the concentrations of embryonic ABA in loblolly pine were found to be similar to or greater than those found in most of the cereals, legumes, and woody angiosperms studied, but the influence of a dissection-induced increase in ABA has not been reported for angiosperms. Angiosperm tissues might be easier to dissect with minimal damage in comparison to conifer, so one might conclude that the increase in ABA due to dissection would be less. There does not appear to be any other commonalities between those species with embryonic ABA concentrations similar to loblolly pine.

Comparing the range and peak endosperm ABA concentrations to the loblolly pine megagametophytic tissues, most species had substantially less ABA (as low as 0.0002 μg/g dry-weight) (33,67,81,90), and only alfalfa (73) had significantly greater concentrations (7-53 μg/g dry-weight with peak value of 130 μg). Therefore, similar to the case for embryonic tissues, ABA concentrations appeared to be greater in the megagametophytes of loblolly pine than in the corresponding tissues (endosperms) of cereals and angiosperms. However, it must be remembered that although these tissues have similar functions, they are very different genetically, i.e., 1*n* in megagametophytes versus 2*n* chromosomes in endosperms. Why the endosperm and embryo ABA concentrations in alfalfa far exceeded all other species is unknown, but might be due in part to use of an invalidated ELISA.

During 1993, loblolly pine embryo tissues displayed the following major increases in ABA on a g fresh-weight basis: very rapid rise from Stage 2 (0.22 μg @ 81% moisture) to Stage 7 (2.64 μg @ 70% moisture); a quick rise from Stage 9A (0.65 μg @ 62% moisture) to Stage 9B (0.96 μg @ 58% moisture); and a slow increase from Stage 9D (0.21 μg @ 24.5% moisture) to Stage 9F (0.84 μg @ 24.5% moisture) (Fig. 25eee and 31B). At seed maturity (9Δ), embryos contained 0.71 μg @ 5.8% moisture. On a μM concentration basis (assuming ABA is uniformly distributed throughout the available water in the tissue) (Fig. 37), the Stage 7 peak denoted an increase of 14 μM, the peak at Stage 9B signifies an increase of 6 μM, Stage 9F an increase of 13 μM, and at 9Δ, an increase of 47 μM. Megagametophytic ABA was highest immediately after fertilization (0.94 μg @ 89.2% moisture). Whole ovules one week prior to fertilization contained 0.13 μg ABA @ 91% moisture. On a μM concentration basis, this peak signified an increase of 3.5 μM at Stage 1A. The megagametophytic ABA

concentration at seed maturity was 10.5 μM, the highest level during development. Therefore, the embryonic ABA peak at Stages 7, 9B, 9F (cone maturity) and 9Δ (seed maturity); and the megagametophytic peak at Stage 1A and seed maturity were the only physiologically significant peaks during loblolly pine embryogenesis as assessed for the 1993 growth season. The very high concentration of ABA in the driest tissues, i.e., late development to cone and seed maturity, may play a role in desiccation tolerance, dormancy, and inhibition of reserve mobilization in loblolly pine seeds. There was insufficient information available to determine the significance of the major embryonic and megagametophytic peaks during 1992.

ABA Distribution and Translocation in Tissues

The principle findings of this investigation of ABA distribution during loblolly pine embryogenesis and development were:

1) on a g dry-weight basis, embryonic ABA was greater than megagametophytic ABA as of Stage 3 during 1993 (Fig. 30, 31, and 35B),

2) and on a per organ basis:

A) ABA in the megagametophytes was consistently higher than in the embryos,

B) the initiation of the embryonic ABA increase generally occurred at the time of the first megagametophytic ABA drop (Fig. 31, 33, and 38),

C) the embryonic ABA peak generally occurred concurrently with the first megagametophytic ABA peak ("early peak") (Fig. 31, 33, and 36B), and

D) embryonic ABA increased (UC_{92} and WB_{92}), remained constant (WA_{92}),

or dropped (WV_{92} and WA_{93}) during the second megagametophytic ABA peak ("late peak") (Fig. 31, 33, and 36B).

The distribution of ABA throughout the reproductive tissues (embryo, endosperm, testa, and seed pod) during development has been studied in many species, but few, if any, generalizations that can be derived from the available data. The ABA concentration in the embryos of tomato (25) and barley (33) were found to be greater than in the respective endosperms, but in maize (67) and alfalfa (73), the opposite was true. In peach (90), embryo and endosperm ABA levels were similar. In soybean (21,41,76), tomato (25), alfalfa (73), pea (74), and bean (75), embryonic ABA exceeded levels found in the testa (seed coat), but, in bean (88) and peach (90), testa ABA concentrations were greater than embryonic ABA. Schussler et al. (70) found that the testa ABA exceeded embryonic ABA in nine genotypes of soybean.

When ABA concentrations were compared within embryo tissues, i.e., cotyledons versus embryonic axes of soybean, Quebedeaux et al. (21) and Van Onckelen (76) found that the cotyledons contained a greater concentration of ABA (>99%) than the embryonic axis, but Hein et al. (41), studying the same species, found the reverse. In Norway maple (91), 80-85% of the ABA was found in the cotyledons, while 15-20% came from the embryonic axis. In bean (77), up to 75% of the ABA was found in the cotyledons. Therefore, the distribution of ABA within the seed tissues during embryogenesis and development appears to vary between, and even within, species.

In loblolly pine, it is difficult to interpret whether the embryonic ABA results primarily from *de-novo* synthesis or import from an external source, i.e., megagametophyte or directly from the mother plant. The initial increase in embryonic ABA generally occurred at the time of the first megagametophytic ABA drop, suggesting that the embryos may import ABA from the megagametophytes. Given that no direct vascular connection exists between the developing embryo and the rest of the mother plant, it has been suggested that the embryo takes all of its water and nutrients directly from the megagametophyte tissues (179). The rates of transfer for water and nutrients are likely to be greatest during early tissue development when moisture levels are high and the tissues are in intimate contact. During later stages of development, when desiccation is rapid, the outer cell layers of the embryo and inner layers of the megagametophyte dry out and break close contact, thus, restricting the movement of water and nutrients. This might explain the loss of synchronization in ABA levels between the two tissues late in development.

After the initial drop in megagametophytic ABA, levels in both tissues increased concurrently with the embryonic ABA levels reaching a peak just prior to the peak in megagametophyte ABA (Figs. 31, 33, and 38). This would suggest that either the tissues are synthesizing their own ABA, or that the maternal plant is exporting ABA to the megagametophyte which is, in turn, exporting ABA to the embryo. Later in development, when desiccation breaks the contact between the embryo and megagametophyte, the embryo probably produces its own ABA.

Little is known about the translocation of ABA within seed tissues. Hendrix et al.

(180) suggested that an important factor in ABA translocation in cotton seeds was a pH differential between the seed coats and the embryo. The seed coats had up to 1.4 pH units more acid than the developing embryos, resulting in a diffusion of ABA from the seed coat to the embryo. In loblolly pine (181), pH changes in the embryo and megagametophyte were recorded during embryogenesis and, although the technique utilized was crude (pressing freshly cut surfaces to pH paper), it suggested that there was approximately a 3.0 pH differential between the embryo and corrosion cavity during the period of 14 to 49 DSF. At ~pH 4, the corrosion cavity appeared more acidic than the embryo (~pH 7), as well as the bulk of the megagametophyte (pH 6 throughout development). On a g dry-weight basis, there was a significant drop in megagametophytic ABA (Fig. 35A) from DSF 14 to 42 followed by a significant increase in embryonic ABA over approximately the same period (Fig. 35B). Therefore, similar to the suggestion by Hendrix et al. (180), changes in tissue pH during development may also play a critical role in the movement of ABA in loblolly pine seeds.

In conclusion, it appears that the maximum ABA concentration changes with respect to localization between the embryo and megagametophyte tissues, and its regulatory role in reproductive development probably differs for each tissue. The concentration changes also suggest that there are different rates of biosynthesis, translocation, and/or metabolism within each tissue (21). The data suggest that the embryo imports ABA from the megagametophyte early in development, but synthesizes its own ABA during late development.

ABA in Suspensor Tissues

Suspected functions of the suspensors include the synthesis and secretion of

hormones to the developing embryo, as well as the conduction of compounds synthesized in other parts of the seed or mother plant to the embryo (182). Alpi et al. (183) discovered concentrations of GAs (GA_1, GA_4, GA_5, GA_6, and GA_{44}) in the suspensors of runner bean that were almost 10-fold greater than those found in the embryo. There is only one study to date that examined the ABA levels in suspensors during embryogenesis. Perata et al. (88) found in runner bean that the suspensor ABA levels were low on a fresh-weight basis during early to mid-development. During mid-to-late development, ABA showed a biphasic distribution peaking at levels almost equal to those found in the embryos at the corresponding stages of development. It has also been shown that during *in-vitro* culture of runner bean embryos, attached suspensors strongly affect late stage embryo development (184).

Unfortunately, very little information can be derived from the suspensor data for loblolly pine. Contamination of the extracting solvent destroyed 12 of the 1992 samples; almost half the number of samples available. Difficulty in weighing and transferring the suspensor tissues contributed to high variability in the results. In many cases, ABA was not even detectable with the ELISA. On a dry-weight basis, the suspensor ABA appeared relatively constant (500-1,000 ng) during embryogenesis, but was relatively low in comparison to the embryo and megagametophyte tissues. On a per suspensor basis, there appeared to be high levels of ABA during early development, but these dropped quickly at mid-development, a trend opposite to that found in the embryo. At mid- to late-development, the suspensors degenerated and were likely dead. Unfortunately, the potential function of the suspensor ABA can not be discerned from this data.

Causal Relationships with Endogenous ABA

ABA and Dry-Weight Accumulation

During loblolly pine embryogenesis in 1992 and 1993, the physiologically significant peak content of embryonic ABA (ng/embryo) appeared to occur prior to and not concurrent with the physiological events of dry-weight accumulation in the embryo (Fig. 40 and 41). After the ABA peak dropped, dry-weight accumulation persisted for an additional 4-6 weeks, at which time the cones were mature. This causal relationship is further supported by the ABA trends on a dry-weight basis. When comparing the 1992 trends (Figs. 30 and 40) and 1993 trends (Figs. 35B and 41), the major embryonic ABA peak (g dry-weight) also occurs prior to and not concurrent with the physiological events of dry-weight accumulation in the embryo. This suggest a causal relationship between ABA and dry-weight accumulation in which it appears that high contents of ABA may initiate the production of reserve proteins in the embryo, but are not necessarily required for their continued synthesis. A similar relationship between ABA and grain filling was proposed for maize by Jones and Brenner (67). They suggested that the peak in ABA may act as a "trigger", rather than as a factor which must constantly be present to mediate filling. ABA acting as a trigger of physiological events during embryogenesis has also been suggested to regulate dormancy (46). On the other hand, the continued increase in dry-weight after ABA levels declined might signal decreased sensitivity to ABA during latter half of embryogenesis in some species (10,48,82, 97).

In general, it has been observed that the amount of ABA increases in parallel with dry- and/or fresh-weight accumulation, with the highest ABA concentrations occurring during the most active growth period in embryos of soybean (41), pea (74), tomato (80), and peach (90); and whole seeds of soybean (21,68), tomato (25), wheat (54,62), barley (66), *Triticale* (71), rice (72), pea (74), sunflower (82), and pear (84). Conflicting with these reports are studies with similar species that show no causal relationships: soybean (70), wheat (33,64), barley (33), and Norway maple (91). Still, in a great number of species, it appears that ABA stimulates growth and protein accumulation during the early to middle phases of embryogenesis (causal evidence). This relationship does not seem to conform to the growth inhibitory role typically assigned to ABA (75), although it is becoming very apparent that ABA coordinates several aspects of embryogenesis by acting in both an inhibitory and stimulatory fashion at different times during embryo development (10,46,60, 68,69). Hsu (75) suggests two hypotheses that could account for the high ABA levels during rapid seed growth: 1) a high degree of ABA comparmentation inside the cells that renders it unable to interfere with growth; and 2) some growth processes may not be inhibited by ABA. Presently, there is no evidence for the first hypothesis. As for the second theory, it is known that for many somatic systems that tissues become insensitive to ABA over time, suggesting that at some point the tissues may no longer respond to ABA at all (10). Therefore, without additional evidence, one can only speculate that ABA controls dry-weight accumulation. There is likely to be a series of additional factors that modulate this control.

ABA and Desiccation

During loblolly pine embryogenesis in 1993, the physiologically significant peak level of embryonic ABA (ng/g dry-weight) appeared to occur prior to and not concurrent with physiological events of desiccation (mg water) in the embryo (Fig. 42B). Megagametophytic ABA, on the other hand (Fig. 43A), dropped rapidly soon after fertilization; a trend similar to that found for the percent moisture content of both the megagametophyte and the embryo. This suggests a causal relationship between megagametophytic ABA concentration and the desiccation of the embryo and megagametophyte. Could the ABA concentration in the megagametophyte initiate and maintain the rate of drying in both tissues?

In general, ABA concentration has been observed to parallel the moisture content of seeds and embryos with rapid desiccation occurring concurrent with the ABA maxima in soybean (21,68), tomato (25), wheat (54,62), *Triticale* (71), and bean (75). This apparent causal relationship has lead many researchers to suggest initiation of desiccation as a putative role of ABA. However, this association was not seen in loblolly pine, nor in wheat (64), rice (72), and sunflower (82). King (62) suggested the opposite relationship: the drying out of tissues may control the ABA content since it is known that drying enhances ABA destruction (185). Hsu (75) suggested that ABA synthesis might be controlled by the change in critical threshold levels of water potential within the embryo. As the potential decreases, so does ABA synthesis.

ABA and Dormancy

During 1993 loblolly pine embryogenesis, ABA on a μM basis was found to greatly increase at seed maturity (9Δ) when the embryo obtains dormancy (Fig. 37). Loblolly pine exhibits moderate to extreme dormancy, requiring 30-60 days of stratification before germination will occur (188). A similar increase was found to occur in the megagametophytic ABA at seed maturity (Fig. 37). This would suggest a causal relationship between embryonic and megagametophytic ABA concentration and the dormancy of the embryo in loblolly pine. High concentrations of ABA in the seeds of bean (76) have been associated with dormancy. Germination was not possible until endogenous ABA concentrations began decreasing, and as ABA decreased, the lag time for germination shortened (Fig. 4). In immature sunflower embryos, germination would occur when ABA contents were low, but dormancy was established immediately after endogenous ABA contents peaked and dropped. High levels of endogenous ABA has also been correlated with the suppression of precocious germination in the embryos of wheat (63), bean (77), apple (89), and Norway maple (91).

Comparison of Zygotic Versus Somatic ABA Levels in Loblolly Pine

Presently, there is one published report on endogenous ABA levels in conifers during zygotic embryogenesis (186) and one on endogenous ABA in loblolly pine somatic embryogenesis by Johns et al. (187). They measured the ABA concentrations in Stage 2-3 somatic embryos maintained on media with ABA and Stage 1-2 embryos maintained on media without ABA. Embryonic ABA concentrations in these two systems were 31 and 18

µg/g fresh-weight, respectively. This compares to 0.2 µg/g fresh-weight for zygotic Stage 2 embryo + suspensors found in seeds (Appendix 1). Thus, embryos from the somatic system contained 90 to 155 times the amount of ABA found in the zygotic system during early development. This suggests that the somatic system is under stress, causing the cells to synthesize high concentrations of ABA. These early somatic embryos fail to develop further suggesting that a high level of ABA early in development is inhibitory to the development of mature somatic embryos. Myers et al. (117) found that elevated levels of ABA during early embryogenesis in isolated zygotic maize kernels resulted in a reduced rate of cell division in the endosperm, and that the nuclear size and starch granule number appeared to be somewhat negatively affected. Although the final embryo dry weight was unaffected in maize, high levels of ABA during conifer somatic embryogenesis may not be optimal, or even desirable.

CONCLUSIONS

A number of conclusions can be derived from these studies with loblolly pine zygotic tissues. These conclusions will be discussed in the following order: analytical methods, endogenous ABA trends, causal relationships, and morphological/physiological changes in embryo development.

ANALYTICAL METHODS

1) The ABA indirect ELISA, using commercially available anti-(+)-ABA mAb and amplified using a streptavidin-biotin-multiple horseradish peroxidase detection system, has been shown to give reliable and sensitive estimates (2-8 pg/100 μL) for ABA while conserving mAb usage.

2) A GC/MS-based standard isotopic dilution assay using enriched stable isotope $^{2}H_{6}$-ABA has been developed that has been shown to give reliable and sensitive estimates (12 pg/μL) for ABA.

3) The ABA indirect ELISA has been validated using definitive GC/MS-SIM at three stages of development: 4 (early), 8B (middle), and 9 (late). This was a critical step as ELISA interfering substances or cross-reactive immunoactive substances might be present in one harvest extreme, but not in the other. This is the first report of such a thorough validation of developing zygotic tissues. Further proof that immunoreactants and/or cross-reactants were not present in the seed extracts was shown by additivity and parallelism techniques.

4) Loblolly pine zygotic tissues were free of immunoactive substances and required

single passage through a 0.45 μm nylon filter for adequate purification. Nylon filtering was also found satisfactory for GC/MS analysis.

5) Endogenous ABA concentrations were significantly increased (~50%) by separation of embryo, suspensor, and megagametophyte tissues during dissection, but were apparently unaffected by washing of embryo and suspensor tissues. Loss of turgor pressure or cell volume may have triggered this increase in ABA biosynthesis. This is the first report regarding the effects of seed dissection on ABA concentra tions.

6) Despite stringent tissue storage conditions (lyophilized; -80°C; nitrogen/vacuum atmosphere; darkness), ABA concentrations were significantly increased (~28%) after 18 days, but remained unchanged after that. This increase may have been the result of stress-induced synthesis of ABA or the hydrolysis of ABA-conjugates. This is the first report on the effects of tissue storage on endogenous ABA concentrations.

7) The procedure of bulking similar stages of development was based on the assumption that ABA was related to visual morphological changes in the embryo. Variability in ABA estimates tended to be highest in the bulked tissues suggesting that ABA fluctuations during embryogenesis were not a function of morphology. Linear time (DSF) may be the best scale for measuring fluctuations of ABA during zygotic development.

ENDOGENOUS ABA TRENDS

Endogenous (+)-ABA levels were measured in the zygotic tissues of loblolly pine during the 1992 and 1993 growing seasons. This is the first report of such a study in conifers.

1) Endogenous ABA in whole seeds showed two peaks (ng/g dry-weight); the first major peak was attributed to megagametophytic ABA and the second to embryonic ABA.

2) Embryonic ABA (dry-weight and embryo basis) trends were similar to those found in any non-coniferous species, i.e., relatively low quantities during early development followed by a maximum during middle to late embryogenesis, and finally dropping to a low level during maturation. This result is remarkable given the fact that modern seed development between conifers and angiosperms arose from separate evolutionary lines as far back as 350 million years ago (10). These results suggest a factor common to all plant species' genetic make-up, despite intra-species and inter-species variations.

3) Megagametophytic ABA trends (dry-weight) were similar to a few non-coniferous species, i.e., high levels very early in development followed by a decline at maturity. On a per megagametophytic basis, loblolly pine showed no similarities to the literature, i.e., the overall trend was complex showing a series of rises (three) and falls throughout development.

4) Embryonic ABA trends from a single mother tree during the 1992 and 1993 growing seasons were almost identical on a days since fertilization (DSF) basis.

Moreover, ABA trends for the four mother trees during the 1992 growing season were also similar. This suggests that environmental factors (such as day length) that are common across the geographic regions are critical in regulating endogenous ABA levels. Moreover, it also appears likely that within-species genetic variations play a minor role. Trends for megagametophytic ABA were similar in the number of peaks and valleys but occurred at different times during development.

5) Little information can be derived from the suspensor data as contamination of the extracting solvent destroyed over half the available samples. On a suspensor basis, ABA appears highest in early tissues, but then drops quickly at mid-development.

6) Zygotic embryonic ABA levels are approximately 90-150 times lower than in corresponding somatic embryos of loblolly pine suggesting that the somatic embryogenesis system may be highly water-stressed early in development. The high level of ABA during Stage 1-3 somatic embryos may be the reason for the low levels of reserve products synthesized in these embryos.

CAUSAL RELATIONSHIPS

Several causal relationships can be suggested from endogenous ABA levels and physiological changes in the zygotic tissues of loblolly pine.

1) During embryogenesis in 1992 and 1993, the major embryonic peak ABA (dry-weight and embryo basis) appeared to occur prior to the accumulation of dry-weight in the embryo. This suggests that high concentrations of ABA may initiate

the production of reserve products in the embryo, but are not necessarily required for their continual synthesis.

2) During the 1993 embryogenesis period, megagametophytic ABA concentrations dropped rapidly soon after fertilization, as did the percent moisture content of both the megagametophyte and embryo. This suggests a causal relationship between the megagametophytic ABA and initiation and control of the rate of desiccation in both tissues.

3) It is difficult to interpret whether the embryonic ABA results primarily from *de-novo* synthesis or import from an external source, i.e., megagametophyte or mother plant. The initial increase in embryonic ABA content generally occurred at the time of the first megagametophytic ABA drop, suggesting that the embryo may import ABA from the megagametophyte. When desiccation is rapid late in development, the outer surfaces of the embryo and megagametophyte dry out and break close contact, possibly restricting the movement of ABA. This would explain the lack of embryo response to changes in megagametophytic ABA during maturation.

4) On a μM basis (1993), embryonic ABA rose sharply during late development reaching a maximum of approximately 47 μM when fully air-dried (9Δ), i.e., dormant. This suggests that a high concentration of ABA may be correlated with the suppression of precocious germination, and the induction and maintenance of dormancy in the embryos of loblolly pine (loblolly pine has a moderate-to-extreme dormancy (188)). A rise is megagametophytic ABA concentration also occurred

after seeds where fully air-dried.

MORPHOLOGICAL/PHYSIOLOGICAL CHANGES IN EMBRYO DEVELOPMENT

1) The estimated fertilization dates for the mother trees were within three days of each other. This suggests that environmental factors (such as day length) that are common to the geographic regions may play a critical role in the timing of fertilization.

2) Despite similar fertilization dates between the four mother trees, cone maturation varied widely (up to 2-3 weeks). This suggests that faster maturing seeds may not necessarily be a function of the time of fertilization, but are probably more dependent on genetic background and/or local environment.

3) Embryos and megagametophytes began drying immediately after fertilization with both tissues displaying similar curvilinear drying patterns. The megagametophytic tissues began drying earlier, and at a faster rate, than the embryos.

4) Embryos displayed an exponential increase in dry-weight accumulation at mid-development (approximately 50 DSF).

FUTURE RESEARCH

The conclusions drawn from this study and the techniques developed for the analysis of ABA suggest two paths for continued research. First, the ABA results for the zygotic system should provide a useful model for optimizing somatic embryogenesis in loblolly pine. The changes in ABA during somatic embryogenesis should be studied further for comparison with zygotic embryogenesis in an effort to discern major differences and give insights into media alterations that could lead to a superior somatic embryogenesis system.

A second direction would be greater in-depth studies on the influence of ABA on gene regulation covering all developmental events from initiation (fertilization) to germination in both somatic and zygotic embryogenesis. The ELISA technique should be adaptable for analyzing other PGHs, and perhaps other major metabolites during development. Such determinations, in conjunction with current technologies, i.e., PCR, southern blotting, electrophoresis, etc., would provide much stronger foundation of fundamental knowledge for improving the current somatic embryogenesis systems. Comparing zygotic and somatic embryogenesis is important for our understanding of the controlling factors in embryogenesis.

Other areas of study could include: 1) determining the relationship between osmotic potential and ABA since both appear to influence physiological events in similar (and dissimilar) fashions; 2) use of gold-labeled antibodies to immunolocalize ABA (or other PGHs) in the seed tissues; 3) quantify ABA within sub-tissues of the embryo, i.e., cotyledons and axis; 4) quantitate other PGHs during zygotic embryogenesis; 5) determine how various PGHs interact in the somatic embryogenesis system, and 6) determine the implications of

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APPENDIX 1

TRANSFORMED DATA OF ENDOGENOUS ABA LEVELS IN ZYGOTIC TISSUES FROM FOUR MOTHER TREES

A19 WB_{92} - Embryonic ABA/ g dry-weight

A20 WB_{92} - Embryonic ABA/ g fresh-weight & organ

A21 WB_{92} - Megagametophytic ABA/ g dry-weight

A22 WB_{92} - Megagametophytic ABA/ g fresh-weight & organ

A23 WB_{92} - Suspensor ABA/ g dry-weight

A24 WB_{92} - Suspensor ABA/ g fresh-weight & organ

A25 WA_{93} - Embryonic ABA/ g dry-weight

A26 WA_{93} - Embryonic ABA/ g fresh-weight & organ

A27 WA_{93} - Megagametophytic ABA/ g dry-weight

A28 WA_{93} - Megagametophytic ABA/ g fresh-weight & organ

A29 WA_{93} - Embryo + suspensor & whole ovule/ g dry-weight

A30 WA_{93}- Embryo + suspensor & whole ovule/ g fresh-weight & organ

Table A1. UC_{92} - embryonic ABA/ g dry-weight

Sample I.D.	Est. Days Since Fertilization	Tissue Wt., g DW/organ	ELISA Results, ng/g dry weight			
			Sample size	Mean	Standard Deviation	95% Conf Lmt.
3C/G	41	---	4	0.06	0.01	0.02
4G	47	$1.8x10^{-5}$	4	2550	223	354
5C/G	49	$2.0x10^{-5}$	9	1504	658	470
6C/G/K	51	$4.5x10^{-5}$	9	1726	317	243
7	53	$4.2x10^{-5}$	5	2366	238	295
8G/K	55	$9.6x10^{-5}$	12	1582	401	255
8BK/BN	62	$3.9x10^{-4}$	12	1487	299	189
9N	69	$7.2x10^{-4}$	12	1882	177	112
9R	73	$9.0x10^{-4}$	27	1301	561	222
9V	80	$1.1x10^{-3}$	28	845	512	198
9Y	87	$1.4x10^{-3}$	21	857	132	61
9iii	94	$1.6x10^{-3}$	20	454	131	61
9vii	101	$1.6x10^{-3}$	28	674	244	95
9x	108	$1.8x10^{-3}$	19	520	216	104
9xii	115	$1.8x10^{-3}$	20	358	108	51
9xv	122	$1.5x10^{-3}$	12	578	163	104
Dried[c]	---	3.2×10^{-3}	20	212	83	39

Table A2. UC_{92} - embryonic ABA/ g fresh-weight & organ

Sample I.D.	Percentage Moisture	Calculated ELISA Results, ng			
		per fresh weight		per embryo	
		Mean	95% Confidence Limits	Mean	95% Confidence Limits
3C/G	N.D.	---	---	0.06	0.02
4G	N.D.	---	---	0.05	0.01
5C/G	N.D.	---	---	0.03	0.001
6C/G/K	N.D.	---	---	0.08	0.01
7G/K	N.D.	---	---	0.10	0.01
8G/K	N.D.	---	---	0.15	0.02
8BK/BN	N.D.	---	---	0.58	0.07
9N	62.0	715	42	1.35	0.08
9R	57.0	559	96	1.17	0.20
9V	44.0	473	112	0.93	0.22
9Y	35.0	382	39	0.84	0.09
9iii	34.0	300	41	0.72	0.10
9vii	30.0	472	66	1.10	0.16
9x	32.0	354	70	0.92	0.18
9xii	31.0	247	35	0.63	0.09
9xv	30.0	405	72	0.87	0.16
Dried	5.7	225	41	0.32	0.06

Table A3. UC_{92} - megagametophytic ABA/ g dry-weight

Sample I.D.	Est. Days Since Fertilization	Tissue Wt., g DW/organ	ELISA Results, ng/g dry weight			
			Sample size	Mean	Standard Deviation	95% Conf. Lmt.
3CCC/GGG	41	$1.6x10^{-3}$	9	1728	474	364
4CCC/GGG	47	$1.7x10^{-3}$	11	1520	332	223
5CCC/GGG	49	$1.9x10^{-3}$	9	1192	271	208
6GGG/KKK	51	$3.4x10^{-3}$	20	793	212	100
7CCC/GGG/KKK	53	$2.8x10^{-3}$	12	869	187	119
8GGG/KKK	55	$3.8x10^{-3}$	12	409	94	59
8BKKK/BNNN	62	$5.0x10^{-3}$	18	482	77	39
9NNN	69	$6.7x10^{-3}$	14	490	62	35
9RRR	73	$7.5x10^{-3}$	12	480	98	62
9VVV	80	$8.1x10^{-3}$	15	267	42	23
9YYY	87	$1.0x10^{-2}$	9	237	34	56
9iii*	94	$1.2x10^{-2}$	20	188	72	34
9vii*	101	$1.1x10^{-2}$	20	230	45	21
9x*	108	$1.3x10^{-2}$	20	199	82	38
9xii*	115	$1.2x10^{-2}$	39	158	50	16
9xv*	122	$1.1x10^{-2}$	89	129	30	7
Dried	---	1.1×10^{-2}	20	42	25	11

Table A4. UC_{92} - megagametophytic ABA/ g fresh-weight & organ

Sample I.D.	Percentage Moisture	Calculated ELISA Results, ng			
		per fresh weight		per organ	
		Mean	95% Conf. Lmt.	Mean	95% Conf. Lmt.
3CCC/GGG	N.D.	---	---	2.68	0.57
4CCC/GGG	N.D.	---	---	2.65	0.39
5CCC/GGG	N.D.	---	---	2.25	0.39
6GGG/KKK	N.D.	---	---	2.67	0.33
7CCC/GGG/KKK	N.D.	---	---	2.43	0.33
8GGG/KKK	N.D.	---	---	1.56	0.23
8BKKK/BNNN	N.D.	---	---	2.39	0.19
9NNN	42.2	283	20	3.27	0.24
9RRR	38.4	296	39	3.60	0.46
9VVV	31.4	183	16	2.16	0.19
9YYY	30.2	165	18	2.44	0.27
9iii*	27.5	136	76	2.22	0.39
9vii*	24.5	174	16	2.54	0.23
9x*	23.8	152	29	2.50	0.48
9xii*	24.6	119	12	1.3	0.20
9xv*	25.0	172	9	1.46	0.08
Dried	5.7	45	12	0.48	0.12

Table A5. UC_{92} - suspensor/ g dry-weight

Sample I.D.	Est. Days Since Fertilization	Tissue Wt., g DW/organ	ELISA Results, ng/g dry weight			
			Sample size	Mean	Standard Deviation	95% Confidence Limits
3CC/GG	41			Contam.		
4CC/GG	47	$3.3x10^{-5}$	12	1184	294	187
5GG	49	$2.4x10^{-5}$	5	682	500	620
6CC/GG/KK	51	---	---	Contam.	---	---
7CC/GG/KK	53	---	---	N.D.	---	---
8GG/KK	55	$2.4x10^{-5}$	12	483	181	114
8BKK/NN	62	$2.4x10^{-5}$	9	516	95	72

Table A6. UC_{92} - suspensor/ g fresh-weight & organ

Sample I.D.	Percentage Moisture	Calculated ELISA Results, ng			
		per fresh weight		per suspensor	
		Mean	95% Confidence Limits	Mean	95% Confidence Limits
3CC/GG	N.D.	---	---	Contam.	---
4CC/GG	N.D.	---	---	0.04	0.005
5GG	N.D.	---	---	0.016	0.015
6CC/GG/KK	N.D.	---	---	Contam.	---
7CC/GG/KK	N.D.	---	---	n.d.	---
8GG/KK	N.D.	---	---	0.013	0.003
8BKK/NN	N.D.	---	---	0.012	0.002

Table A7. WV_{92} - embryonic ABA/ g dry-weight

Sample I.D.	Est. Days Since Fertilization	Tissue Wt., g DW/organ	ELISA Results, ng/g dry weight			
			Sample size	Mean	Standard Deviation	95% Confidence Limits
3B	36	---	8	0.02	0.004	0.003
4B/F	41	$4x10^{-6}$	9	6442	1540	1183
5B/F	43	$9x10^{-6}$	8	4775	2035	1696
6F/J	45	$1.7x10^{-5}$	10	1579	898	642
7B/F/J	47	$6.2x10^{-5}$	20	1355	561	263
8F/J	50	$9.0x10^{-5}$	20	2132	341	160
8BJJJ	54	$1.9x10^{-4}$	26	4632	1661	671
9Q	68	$7.0x10^{-4}$	37	1463	532	182
9U	75	$9.5x10^{-4}$	40	1155	271	87
9X	82	$1.2x10^{-3}$	40	716	149	48
9ii	89	$1.4x10^{-3}$	30	683	215	80
9vi	96	$1.3x10^{-3}$	39	832	231	75
9xi	110	$1.5x10^{-3}$	38	449	208	69
9xiv	117	$1.2x10^{-3}$	37	125	78	26

Table A8. WV_{92} - embryonic ABA/ g fresh-weight & organ

Sample I.D.	Percentage Moisture	Calculated ELISA Results, ng			
		per fresh weight		per embryo	
		Mean	95% Confidence Limits	Mean	95% Confidence Limits
3B	N.D.	---	---	0.02	0.003
4B/F	N.D.	---	---	0.03	.01
5B/F	N.D.	---	---	0.04	.02
6F/J	N.D.	---	---	0.03	.01
7B/F/J	N.D.	---	---	0.09	0.02
8F/J	N.D.	---	---	0.19	.01
8BJ	N.D.	---	---	0.89	0.13
9Q	59.0	600	74	1.02	0.13
9U	42.0	670	56	1.10	0.09
9X	33.0	480	31	0.75	0.06
9ii	32.0	464	55	0.95	0.11
9vi	32.0	566	51	1.05	0.10
9xi	31.0	310	47	0.65	0.10
9xiv	33.0	84	17	0.15	0.03

Table A9. WV_{92} - megagametophytic ABA/ g dry-weight

Sample I.D.	Est. Days Since Fertilization	Tissue Wt., g DW/organ	ELISA Results, ng/g dry weight			
			Sample size	Mean	Standard Deviation	95% Confidence Limits
3BBB	36	$9.3x10^{-4}$	40	2308	722	231
4BBB/FFF	41	$1.1x10^{-3}$	30	1536	390	145
5BBB/FFF	43	$1.5x10^{-3}$	30	1146	200	75
6BBB/FFF/JJJ	45	$1.6x10^{-3}$	40	1176	808	258
7BBB/FFF/JJJ	47	$1.6x10^{-3}$	39	1687	710	231
8FFF/JJJ	50	$2.3x10^{-3}$	39	866	215	70
8BJJJ	54	$2.4x10^{-3}$	41	337	103	32
9QQQ	68	$4.8x10^{-3}$	39	730	234	76
9UUU	75	$6.0x10^{-3}$	38	408	149	49
9XXX	82	$6.7x10^{-3}$	29	280	77	30
9ii*	89	$8.5x10^{-3}$	30	254	150	56
9vi*	96	$7.7x10^{-3}$	10	250	35	25
9xi*	103	$8.2x10^{-3}$	29	189	68	26
9xi*	110	$8.9x10^{-3}$	30	400	102	38
9xiv*	117	$8.3x10^{-3}$	39	191	71	23

Table A10. WV_{92} - megagametophytic ABA/ g fresh-weight & organ

Sample I.D.	Percentage Moisture	Calculated ELISA Results, ng			
		per fresh weight		per megagametophyte	
		Mean	95% Confidence Limits	Mean	95% Confidence Limits
3BBB	N.D.	---	---	2.14	0.21
4BBB/FFF	N.D.	---	---	1.63	0.15
5BBB/FFF	N.D.	---	---	1.75	0.11
6BBB/FFF/JJJ	N.D.	---	---	1.90	0.42
7BBB/FFF/JJJ	N.D.	---	---	2.77	0.38
8FFF/JJJ	N.D.	---	---	1.95	0.16
8BJJJ	N.D.	---	---	0.80	0.08
9QQQ	42.8	418	44	3.50	0.37
9UUU	31.5	279	33	2.41	0.29
9XXX	29.9	196	21	1.90	0.20
9ii*	26.9	186	41	2.16	0.48
9vi*	28.0	180	18	1.92	0.20
9ix*	25.7	140	19	1.52	0.22
9xi*	25.9	296	28	3.54	0.34
9xiv*	26.9	140	17	1.59	0.19

Table A11. WV_{92} - suspensor/ g dry-weight

Sample I.D.	Est. Days Since Fertilization	Tissue Wt., g DW/organ	ELISA Results, ng/g dry weight			
			Sample size	Mean	Standard Deviation	95% Confidence Limits
3BB	36	---	6	226	116	121
4BB/FF	41	---	---	N.D.	---	---
5BB/FF	43	---	---	Contam.	---	---
6FF/JJ	45	---	---	N.D.	---	---
7FF	47	---	---	N.D.	---	---
9QQ	68	---	12	1461	273	173

Table A12. WV_{92} - suspensor/ g fresh-weight & organ

Sample I.D.	Percentage Moisture	Calculated ELISA Results, ng			
		per fresh weight		per suspensor	
		Mean	95% Confidence Limits	Mean	95% Confidence Limits
3BB	N.D.	---	---	0.05	0.03
4BB/FF	N.D.	---	---	n.d.	---
5BB/FF	N.D.	---	---	Contam.	---
6FF/JJ	N.D.	---	---	n.d.	---
7FF	N.D.	---	---	n.d.	---
9QQ	N.D.	---	---	0.02	0.002

Table A13. WA_{92} - embryonic ABA/ g dry-weight

Sample I.D.	Est. Days Since Fertilization	Tissue Wt., g DW/organ	ELISA Results, ng/g dry weight			
			Sample size	Mean	Standard Deviation	95% Confidence Limits
3D/H	39	---	10	0.48	0.08	0.06
4D/H	41	$7.0x10^{-6}$	11	9097	1342	901
5D/H	43	---	11	0.08	0.02	0.01
6D/H/L	45	$1.9x10^{-5}$	10	6977	979	700
7D/H/L	46	$3.2x10^{-5}$	22	6144	1077	477
8H/L	48	$9.4x10^{-5}$	18	4953	686	341
8BL/BO	55	$2.5x10^{-4}$	34	2247	450	139
9O	62	$5.9x10^{-4}$	42	1589	390	122
9S	69	$8.0x10^{-4}$	43	729	181	56
9W	76	$1.1x10^{-3}$	19	328	58	28
9Z	83	$1.3x10^{-3}$	44	401	180	55
9iv	90	$1.3x10^{-3}$	33	334	67	24
9viii	97	$1.4x10^{-3}$	29	553	95	37
9xiii	111	$1.4x10^{-3}$	33	770	272	97

Table A14. WA_{92} - embryonic ABA/ g fresh-weight & organ

Sample I.D.	Percentage Moisture, %	Calculated ELISA Results, ng			
		per fresh weight		per embryo	
		Mean	95% Confidence Limits	Mean	95% Confidence Limits
3D/H	N.D.	---	---	0.48	0.06
4D/H	N.D.	---	---	0.06	0.01
5D/H	N.D.	---	---	0.08	0.01
6D/H/L	N.D.	---	---	0.13	0.01
7D/H/L	N.D.	---	---	0.20	0.02
8H/L	N.D.	---	---	0.47	0.03
8BL/BO	N.D.	---	---	0.57	0.04
9O	58.5	651	50	0.94	0.07
9S	49.0	372	28	0.59	0.05
9W	44.0	184	15	0.35	0.03
9Z	39.0	245	33	0.50	0.07
9iv	34.0	220	16	0.43	0.03
9viii	31.0	382	26	0.76	0.05
9xiii	31.0	531	67	1.07	0.14

Table A15. WA_{92} - megagametophytic ABA/ g dry-weight

Sample I.D.	Est. Days Since Fertilization	Tissue Wt., g DW/organ	ELISA Results, ng/g dry weight			
			Sample size	Mean	Standard Deviation	95% Confidence Limits
3DDD/HHH	39	$2.6x10^{-3}$	21	1175	82	37
4HHH	41	$3.0x10^{-3}$	42	451	105	33
5DDD/HHH	43	$2.8x10^{-3}$	22	823	531	235
6DDD/HHH	45	$3.2x10^{-3}$	43	335	61	19
7HHH/LLL	46	$3.8x10^{-3}$	21	517	146	67
8HHH/LLL	48	$4.3x10^{-3}$	43	454	187	58
8BLLL/OOO	55	$5.5x10^{-3}$	43	327	113	35
9OOO	62	$6.2x10^{-3}$	42	571	74	23
9SSS	69	$7/0x10^{-3}$	22	353	50	22
9WWW	76	$8.6x10^{-3}$	41	120	37	11
9ZZZ	83	$9.5x10^{-3}$	44	302	126	39
9iv*	90	$9.7x10^{-3}$	42	203	116	36
9xiii*	97	$1.0x10^{-2}$	12	115	25	16

Table A16. WA_{92} - megagametophytic ABA/ g fresh-weight & organ

Sample I.D.	Percentage Moisture, %	Calculated ELISA Results, ng			
		per fresh weight		per megagametophyte	
		Mean	95% Confidence Limits	Mean	95% Confidence Limits
3DDD/HHH	N.D.	---	---	3.02	0.10
4HHH	N.D.	---	---	1.37	0.10
5DDD/HHH	N.D.	---	---	2.31	0.66
6DDD/HHH	N.D.	---	---	1.08	0.05
7HHH/LLL	N.D.	---	---	1.96	0.25
8HHH/LLL	N.D.	---	---	1.95	0.25
8BLLL/BOOO	N.D.	---	---	1.79	0.19
9OOO	32.2	387	16	3.56	0.14
9SSS	29.6	249	15	2.47	0.16
9WWW	27.6	87	8	1.03	0.10
9ZZZ	25.5	226	29	2.86	0.37
9iv*	22.1	158	28	1.97	0.35
9xiii*	23.0	89	12	1.15	0.02

Table A17. WA_{92} - suspensor/ g dry-weight

Sample I.D.	Est. Days Since Fertilization	Tissue Wt., g DW/organ	ELISA Results, ng/g dry weight			
			Sample size	Mean	Standard Deviation	95% Confidence Limits
3DD/HH	39	$1.3x10^{-4}$	8	2310	958	798
4DD/HH	41	---	---	Contam.	---	---
5DD/HH	43	---	---	Contam.	---	---
6DD/HH/LL	45	$8.6x10^{-4}$	12	143	116	73
7DD/HH/LL	46	---	---	N.D.	---	---
8HH/LL	54	---	---	Contam.	---	---
8BLL/OO	55	$2.2x10^{-3}$	5	53	34	42
9OO	62	$2x10^{-5}$	13	679	263	158
9SS	69	$7.8x10^{-6}$	4	602	224	356

Table A 18. WA_{92} - suspensor/ g fresh-weight & organ

Sample I.D.	Percentage Moisture	Calculated ELISA Results, ng			
		per fresh weight		per organ	
		Mean	95% Confidence Limits	Mean	95% Confidence Limits
3DD/HH	N.D.	---	---	0.30	0.10
4DD/HH	N.D.	---	---	Contam.	---
5DD/HH	N.D.	---	---	Contam.	---
6DD/HH/LL	N.D.	---	---	0.12	0.06
7DD/HH/LL	N.D.	---	---	n.d.	---
8HH/LL	N.D.	---	---	Contam.	---
8BLL/BOO	N.D.	---	---	0.10	0.09
9OO	N.D.	---	---	0.014	0.004
9SS	N.D.	---	---	0.005	0.003

Table A19. WB_{92} - embryonic ABA/ g dry-weight

Sample I.D.	Est. Days Since Fertilization	Tissue Wt., g DW/organ	ELISA Results, ng/g dry weight			
			Sample size	Mean	Standard Deviation	95% Confidence Limits
3A/E	41	---	9	0.64	0.05	0.04
4A/E	43	$1.5x10^{-5}$	10	5473	370	265
5A/E	45	$1.9x10^{-5}$	11	4403	557	373
6A/E	47	$2.1x10^{-5}$	9	2319	429	329
7A/E	50	$6.3x10^{-5}$	21	3118	704	321
8A/E	54	$1.2x10^{-4}$	19	2334	1584	764
8BI	55	$2.0x10^{-4}$	21	1746	541	247
9I	57	$4.8x10^{-4}$	36	1463	289	98
9M	64	$6.2x10^{-4}$	40	823	185	59
9P	71	$8.3x10^{-4}$	39	724	161	52
9T	78	$1.1x10^{-3}$	43	253	135	42
9i	85	$1.3x10^{-3}$	33	527	125	45
9v	92	$1.4x10^{-3}$	39	658	304	99

Table A20. WB_{92} - embryonic ABA/ g fresh-weight & organ

Sample I.D.	Percentage Moisture	Calculated ELISA Results, ng			
		per fresh weight		per embryo	
		Mean	95% Confidence Limits	Mean	95% Confidence Limits
3A/E	N.D.	---	---	0.64	0.04
4A/E	N.D.	---	---	0.08	0.004
5A/E	N.D.	---	---	0.08	0.01
6A/E	N.D.	---	---	0.049	0.01
7A/E	N.D.	---	---	0.20	0.02
8A/E	N.D.	---	---	0.27	0.09
8BI	N.D.	---	---	0.35	0.05
9I	63.4	535	36	0.70	0.05
9M	63.3	302	22	0.51	0.04
9P	49.4	366	26	0.60	0.04
9T	43.6	143	24	0.27	0.04
9i	28.5	377	32	0.68	0.06
9v	30.8	455	69	0.92	0.14

Table A21. WB_{92} - megagametophytic ABA/ g dry-weight

Sample I.D.	Est. Days Since Fertilization	Tissue Wt., g DW/organ	ELISA Results, ng/g dry weight			
			Sample size	Mean	Standard Deviation	95% Confidence Limits
3AAA/EEE	41	$2.3x10^{-3}$	43	1355	534	164
4AAA/EEE	43	$2.9x10^{-3}$	22	760	263	117
5AAA/EEE	45	$2.9x10^{-3}$	43	742	215	66
6AAA/EEE/III	47	$3.0x10^{-3}$	41	555	226	72
7AAA/EEE	50	$3.3x10^{-3}$	43	196	54	17
8AAA/EEE	54	$3.7x10^{-3}$	42	631	141	44
8BI	55	$4.7x10^{-3}$	44	445	144	44
9III	57	$4.8x10^{-3}$	21	633	132	61
9MMM	64	$5.4x10^{-3}$	43	477	116	36
9PPP	71	$6.2x10^{-3}$	33	414	76	27
9TTT	78	$6.5x10^{-3}$	22	205	54	24
9iii	85	$7.6x10^{-3}$	13	234	41	24
9vvv	94	$8.3x10^{-3}$	10	249	17	12

Table A22. WB_{92} - megagametophytic ABA/ g fresh-weight & organ

Sample I.D.	Percentage Moisture, %	Calculated ELISA Results, ng			
		per fresh weight		per megagametophyte	
		Mean	95% Confidence Limits	Mean	95% Confidence Limits
3AAA/EEE	N.D.	---	---	3.12	0.38
4AAA/EEE	N.D.	---	---	2.20	0.33
5AAA/EEE	N.D.	---	---	2.12	0.19
6AAA/EEE/III	N.D.	---	---	1.69	0.21
7AAA/EEE	N.D.	---	---	0.65	0.06
8AAA/EEE	N.D.	---	---	2.33	0.16
8BIII	N.D.	---	---	2.11	0.21
9III	37.4	396	38	3.06	0.29
9MMM	34.8	311	23	2.57	0.20
9PPP	31.7	283	18	2.56	0.16
9TTT	29.1	145	17	1.33	0.15
9iii*	24.1	178	18	1.78	0.19
9vvv*	23.3	191	9	2.10	0.07

Table A23. WB_{92} - suspensor/ g dry-weight

Sample I.D.	Est. Days Since Fertilization	Tissue Wt., g DW/organ	ELISA Results, ng/g dry weight			
			Sample size	Mean	Standard Deviation	95% Confidence Limits
3AA/EE	41	---	---	N.D.	---	---
5AA/EE	45	---	---	Contam.	---	---
6AA/EE/II	47	$1.4x10^{3}$	9	196	107	82
7AA/EE	50	---	---	N.D.	---	---
8AA/EE	55	---	---	Contam.	---	---
9II	57	$1.3x10^{-5}$	11	2329	537	361
9PP	71	$6.3x10^{-6}$	5	436	339	420

Table A24. WB_{92} - suspensor/ g fresh-weight & organ

Sample I.D.	Percentage Moisture	Calculated ELISA Results, ng			
		per fresh weight		per suspensor	
		Mean	95% Confidence Limits	Mean	95% Confidence Limits
3AA/EE	N.D.	---	---	n.d.	---
5AA/EE	N.D.	---	---	Contam.	---
6AA/EE/II	N.D.	---	---	0.27	0.11
7AA/EE	N.D.	---	---	n.d.	---
8AA/EE	N.D.	---	---	Contam.	---
9II	N.D.	---	---	0.03	0.004
9PP	N.D.	---	---	0.0027	0.0026

Table A25. WA_{93} - embryonic ABA/ g dry-weight

Sample I.D.	Est. Days Since Fertilization	Tissue Wt., g DW/organ	ELISA Results, ng/g dry weight			
			Sample size	Mean	Standard Deviation	95% Confidence Limits
3e-6	32	---	5	0.006	0.005	0.003
4e-6	35	$6.8x10^{-6}$	12	1372	635	454
5e-7	42	$1.1x10^{-5}$	8	1972	694	578
6e-7	44	$2.0x10^{-5}$	11	3322	478	321
7e-7/8	47	$4.1x10^{-5}$	12	8792	1047	664
8e-8/9	50	$1.5x10^{-4}$	13	1916	318	192
8Be-9	56	$3.7x10^{-4}$	35	2133	368	126
9e-9	60	$5.0x10^{-4}$	12	1699	204	130
9e-10	63	$5.5x10^{-4}$	51	2276	431	121
9e-11	77	$1.1x10^{-3}$	77	545	175	57
9e-12	91	$1.2x10^{-3}$	91	282	65	21
9e-13	99	$1.2x10^{-3}$	38	540	83	27
9e-14	114	$1.4x10^{-3}$	33	1106	158	56
Dried[c]	---	1.4×10^{-3}	27	673	185	73

Table A26. WA_{93} - embryonic ABA/ g fresh-weight & organ

Sample I.D.	Percentage Moisture	Calculated ELISA Results, ng				per µM
		per fresh weight		per embryo		
		Mean	95% Confidence Limits	Mean	95% Confidence Limits	
3e-6	78.0	---	---	0.006	0.003	---
4e-6	77.0	316	104	0.009	0.003	1.6
5e-7	72.5	542	159	0.02	0.01	2.8
6e-7	71.0	963	93	0.07	0.03	5.1
7e-7/8	70.0	2638	199	0.36	0.03	14.3
8e-8/9	68.0	613	62	0.28	0.08	3.4
8Be-9	64.5	754	44	0.77	0.05	4.4
9e-9	62.0	646	49	0.84	0.07	3.9
9e-10	58.0	956	51	1.24	0.07	6.2
9e-11	37.3	342	36	0.58	0.06	3.5
9e-12	24.5	213	16	0.35	0.03	3.3
9e-13	24.5	408	21	0.65	0.03	6.3
9e-14	24.5	835	42	1.51	0.08	12.9
Dried	5.8	714	78	0.92	0.1	46.7

Table A27. WA_{93} - megagametophytic ABA/ g dry-weight

Sample I.D.	Est. Days Since Fertilization	Tissue Wt., g DW/organ	ELISA Results, ng/g dry weight			
			Sample size	Mean	Standard Deviation	95% Conf. Lmts.
1m-A	7	$4.5x10^{-4}$	45	8682	2005	602
1m-B	14	$4.6x10^{-4}$	48	7173	1738	505
2m	28	$9.0x10^{-4}$	43	3023	530	163
3m-6	32	$1.4x10^{-3}$	42	1336	244	76
4m-6	35	$1.5x10^{-3}$	29	950	117	44
5m-7	42	$2.2x10^{-3}$	38	395	90	30
6m-7	44	$2.6x10^{-3}$	45	360	82	25
7m-7	47	$3.9x10^{-3}$	47	279	70	32
8m-8	50	$4.0x10^{-3}$	50	248	41	14
8Bm-9	56	$5.5X10^{-3}$	56	390	59	19
9m-9	60	$5.4x10^{-3}$	22	447	73	33
9m-10	63	$5.8x10^{-3}$	43	381	54	17
9m-11	77	$8.0x10^{-3}$	33	449	75	26
9m-12	91	$9.1x10^{-3}$	39	161	36	12
9m-13	99	$8.7x10^{-3}$	44	137	42	13
9m-14	114	$9.9x10^{-3}$	42	114	43	13
Dried[c]	---	$9.9x10^{-3}$	29	152	129	49

Table A28. WA_{93} - megagametophytic ABA/ g fresh-weight & organ

Sample I.D.	Percentage Moisture	Calculated ELISA Results, ng				per µM
		per fresh weight		per megagametophyte		
		Mean	95% Confidence Limits	Mean	95% Confidence Limits	
1m-A	89.2	938	65	3.86	0.27	4.0
1m-B	86.9	817	57	3.3	0.23	3.6
2m	77.4	683	37	2.71	0.15	3.3
3m-6	72	374	22	1.92	0.11	2.0
4m-6	68.6	298	14	1.5	0.06	1.6
5m-7	52.7	187	14	0.89	0.07	1.3
6m-7	47	191	13	0.92	0.06	1.5
7m-7	43	159	0.13	1.1	0.13	1.4
8m-8	39.3	151	7	1	0.05	1.5
8Bm-8	37.4	244	11	2.15	0.1	2.5
9m-9	35	291	20	2.43	0.17	3.1
9m-10	33.2	255	11	2.21	0.1	2.9
9m-11	26.2	331	20	3.59	0.22	4.8
9m-12	22.2	125	10	1.47	0.1	2.1
9m-13	22.2	107	10	1.19	0.12	1.8
9m-14	22.2	89	10	1.12	0.13	1.5
Dried	5.8	161	52	1.5	0.48	10.5

Table A29. WA_{93} - embryo + suspensor & whole ovule/ g dry-weight

Sample I.D.	Est. Days Since Fertilization	Tissue Wt., g DW/organ	ELISA Results, ng/g dry weight			
			Sample size	Mean	Standard Deviation	95% Confidence Limits
(1A)e+s	7	$9x10^{-6}$	7	1189	257	238
(1B)e+s	14	$7.5x10^{-6}$	9	871	372	286
(2)e+s	28	$1.1x10^{-5}$	6	1130	729	763
-1wo	-7	$3.8x10^{-4}$	11	3160	581	390
0wo	fertilization	$3.8x10^{-4}$	34	4806	1516	529
(1A)wo	7	$4.8x10^{-4}$	49	7233	1246	358
(1B)wo	14	$4.9x10^{-4}$	33	7243	2472	877
2wo	28	$9.6x10^{-4}$	51	2652	459	129

Table A30. WA_{93} - embryo + suspensor & whole ovule/ g fresh-weight & organ

Sample I.D.	Percentage Moisture	Calculated ELISA Results, ng				per μM
		per fresh weight		per embryo+suspensor or whole ovule		
		Mean	95% Confidence Limits	Mean	95% Confidence Limits	
(1A)e+s	89.0	131	26	0.01	0.002	0.6
(1B)e+s	86.9	114	37	0.01	0.003	0.5
(2)e+s	81.0	215	145	0.012	0.009	1.0
-1wo	91.0	284	35	1.21	0.30	1.2
0wo	90.0	481	53	1.80	0.02	2.0
(1A)wo	89.2	781	38	3.49	0.17	3.3
(1B)wo	86.9	949	115	3.60	0.40	4.1
2wo	77.4	597	29	2.56	0.13	2.9

APPENDIX 2

EFFECTS OF METHANOL IN THE INDIRECT ELISA ABA STANDARDS

INTRODUCTION

Many of the ELISA quantitation analyses revealed deviations from linearity (that is, a "low-dose hook" or concavity at low ABA concentrations) (Fig. A2.1). This non-linearity negatively affected the low-end sensitivity. It was hypothesized that the presence of methanol in all of the ABA standards except for B_0 (no ABA), may have inhibited reaction rates and/or equilibrium during the ELISA. The purpose of this experiment was to determine whether the use of methanol in the make up of the ABA stock solution adversely effected the ELISA calibration curve.

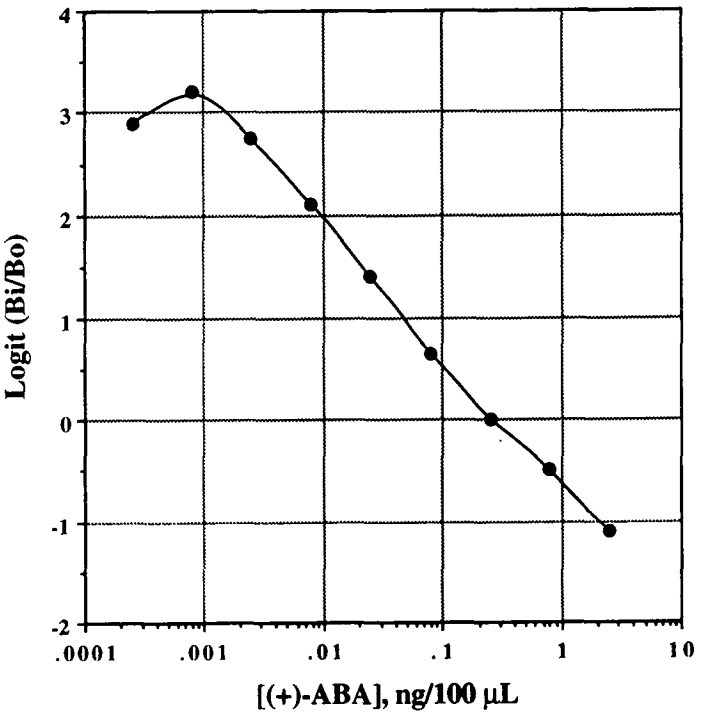

Figure A2.1. Example of a low-dose hook in an ELISA standard curve.

MATERIALS AND METHODS

The materials and methods used in this experiment are described in the ABA Indirect ELISA Materials and Methods Development Section in Chapter 2. Stock solutions of 5,000-6,000 ng/μL *cis*-(+)-ABA were prepared in absolute methanol (standard method) and in assay buffer. Two sets of ABA standards, ranging from 15.8 to 0.0005 ng/100 μL (+)-ABA were made by serial exponential dilution from these stock solutions in assay buffer. The ELISA standard curve ranged from 2.5 to 0.00025 ng/100 μL (+)-ABA. The standard curves for each set of ABA standards were performed on a single microtiter plate to eliminate plate effects.

STATISTICAL ANALYSIS

A two-way ANOVA with fixed effects was conduicted in order to determine which effects significantly influenced the absorbances measured at 450 nm. The effects considered were 1) standard ABA concentration (A), 2) presence of methanol in the standards (B), and 3) the interaction between ABA concentration and the presence of methanol (A x B). Analyses were carried out at an α = 0.05.was used to determine which effects significantly influenced the measured absorbances at 450 nm. Analyses were carried out at an α = 0.05.

RESULTS

Table A2.1 shows the percent methanol (v/v) in each ABA standard used for constructing the ELISA calibration curve (when using the methanolic ABA stock

Table A2.1. Percent methanol in each ABA standard used

in construction of ELISA calibration curve.

ABA Standard, ng/100 μL	Percent Methanol in Standard (v/v), %
7.9 (B_{max})	7.3×10^{-3}
2.5	2.3×10^{-3}
0.79	7.4×10^{-4}
0.25	2.4×10^{-4}
0.079	7.6×10^{-5}
0.025	2.4×10^{-5}
0.0079	7.8×10^{-6}
0.0025	2.5×10^{-6}
0.00079	7.9×10^{-7}
0.00025	2.5×10^{-7}
0 (B_0)	0

solution). The amount of methanol decreased exponentially during the serial dilutions, and there was no methanol in the B_0 standard. Results of the statistical analyses are shown in Table A2.2. As expected, the ABA standard concentrations significantly affected the final absorbances ($\alpha = 0.05$), however, the presence of methanol in the ABA standards did not significantly affect the absorbances ($\alpha > 0.05$). Therefore, the low-dose hook phenomena does not appear attributable to the presence of methanol in the ABA standards. The use of methanol in the ABA stock solution make up appears to be an acceptable practice, and it also has several advantages over using an aqueous buffer: 1) due to ABA insolubility in water, higher concentrations can be made up in organic solvents; 2) higher concentration solutions are more stable than very dilute solutions over a longer period

Table 2A.2. ANOVA for Effects of Concentration of Methanol on ELISA Estimates.

Source of Error	Degrees of Freedom	Sum of Squares	Mean Square	F-ratio	ρ
ABA Concentration (A)	5	154.4	30.9	140.5	0.000[a]
Presence of MeOH (B)	1	0.637	0.637	2.9	0.104
Interaction (AxB)	5	0.322	0.006	0.29	0.911
Error	20	4.397	0.220		
Total	31	142.9			

a: significant at $\alpha = 0.05$.

of time (151); and 3) use of methanol allows storage at -20°C or less without freezing, thereby improving stability. Disadvantages of using methanol would include the possibility of methanolysis and the potential of inhibiting or denaturing the proteins used in ELISA.

One possible cause of the low-dose hook phenomena could be positive cooperativity, an effect occurring when two antibodies are mixed with antigen and form a stable circular complex (17,189). Use of too little or out-of-date tracer could also be a possible cause (17). A more plausible explanation, however, would be one that is unique to solid-phase assays, i.e., when absorbing a protein or protein conjugate (e.g., ABA-BSA) to polystyrene. As the plastic surface approaches protein saturation, stearic interference reduces the area of interaction between the plastic surface and the protein. The binding of an antibody to a "weakly" bound antigen (e.g., ABA-BSA) molecule might result in its desorbtion from the plastic surface. This is more likely to occur when high concentrations of antibody are added, and no sample antigen is present to bind antibody prior to addition to plate (190).

APPENDIX 3

ANALYSIS OF INDOLE-3-ACETIC ACID IN LOBLOLLY PINE SEED TISSUES

INTRODUCTION

In the initial A490 proposal for this dissertation research, it was proposed that indole-3-acetic acid (IAA) fluctuations during loblolly pine zygotic embryogenesis would be included for analysis. Similar to the process used with ABA, methods were developed to extract, purify, and quantitate IAA in loblolly pine seed tissues. Analysis of IAA was later dropped from the A490 thesis objectives because of logistical and pragmatic concerns ragarding development of a workable (in)direct IAA ELISA. Therefore, a GC/MS quantitation method was developed for IAA.

MATERIALS AND METHODS

Materials

Plant, extraction and purification, and GC/MS materials were identical to those described for ABA in the methods sections of Chapter 2. Radioactive (50 μCi) indole-3-acetic acid ([^{14}C]-IAA) (I-8262), IAA (I-2886), and all other indole compounds listed in the following results section were obtained from Sigma (St. Louis, MO). Methyl-IAA was purchased from Fluka Chemika-Biochemika (Buchs, Switzerland). The enriched stable isotope $^{13}C_6$-IAA (#BH-1019R) was obtained from Cambridge Isotope (Woburn, MA). The ^{14}C quench set (P/H 566681) was obtained from Beckman Bioanalytical (Norcross, GA). HPLC-grade acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ).

Methods

The extraction and purification and GC/MS methods were identical to those decribed for ABA in Chapter 2.

HPLC Methods

All HPLC techniques were performed on a liquid chromatograph (Varian Model 5000) using a reverse-phase Econosphere C_{18} HPLC column (described in Chapter Two). The HPLC protocol was developed in order to determine whether any immunoreactive IAA cross-reactants were present in loblolly pine zygotic tissue and, if necessary, to separate them from IAA. All of the indole compounds were methylated using diazomethane (as described in Chapter 2 for ABA). The purified sample was dissolved in 200 μL of 30% acetonitrile (v/v) adjusted to pH 3.0 using acetic acid, prior to injection onto the column. The mobile phase was isocratic with a composition of 30% (v/v) acetonitrile (pH 3.0) and a flowrate of 1.0 mL/minute over a 60 minute interval.

RESULTS

Selection of Extraction Method

The extraction method chosen was that most commonly used in the literature (11,13,14). The extraction solvent was 80% methanol in deionized or RO water containing 25 mg/L BHT, and adjusted to pH 7.0-7.5 (neutral methanolic solution). This was the same solvent used for ABA extraction.

Selection of Purification Method

Table A3.1 displays the percent recovery of ^{14}C-IAA based on acidic and neutral purification solvent trials. Trials Nos. 1 and 2, used acidic solvent without plant tissue and yielded very low recovery rates. Trial Nos. 3 and 4 resulted in excellent recovery of ^{14}C-IAA in the absence of plant tissue. Plant tissue was added for the extraction steps in Trial No. 5; Trial No. 5 showed slightly less recovery than Trial Nos. 3 and 4. This was expected because of the potential for trapping of IAA in the plant tissues. The neutral solvent system was selected for purification because of the high recovery rates obtained. The lack of a significant difference in recovery in Trial Nos. 3-5 suggested that nylon filtering alone was sufficient for purifying the plant tissues (assuming no immunoreactants are present).

Degradation of IAA during Extracting and Purification

HPLC fractionation was used to determine the degree of IAA degradation during extraction and purification and clean-up. Similar to trends found in ABA, the before and after peaks (data not shown) had identical retention times with no additional peaks following extraction and purification. Therefore, there appeared to be no significant degradation of IAA during these procedures.

Table A3.1. Selection of a purification solvent system.

Solvent Type	Trial No.	Plant Tissue?	Purification	% Recovery[a] (± Std. Dev.)
Acidic	1	No	Nylon filter	63 ± 3
			Nylon filter + C_{18} column	57 ± 3
	2	No	Nylon filter	83 ± 4
			Nylon filter + C_{18} column	43 ± 2
Neutral	3	No	Nylon filter	100 ± 5
			Nylon filter + C_{18} column	99 ± 5
	4	No	Nylon filter	100 ± 5
			Nylon Filter + C_{18} column	99 ± 5
	5	Yes	Nylon filter	97 ± 5
			Nylon filter + C_{18} column	89 ± 4

a: Based on scintillation counting of ^{14}C-IAA.

Ascertaining the Degree of Purification Required for GC/MS Quantitation of IAA

IAA-spiked plant extracts were analyzed using GC/MS-SCAN (Fig. 20/Chapter 2) and GC/MS-SIM (Fig. 21/Chapter Two) either as crude extracts, after nylon filtering, or after nylon filtering + preparatory C_{18} column. Nylon filtering alone removed the majority of the background contamination. The addition of the preparatory C_{18} column step did not appear to improve the purity of the extract. Therefore, nylon filtering alone was judged adequate purification for quantifying IAA by GC/MS. Quantiative successive approximation was not

performed for GC/MS.

HPLC Results for Identification of IAA-Monoclonal Antibody Cross-Reactants

Table A3.2 depicts the major immunoreacting indoles, their percent cross-reactivity, and their R_t on the HPLC after methylation. Although loblolly pine extrtacts were not tested, this HPLC system was capable of separating the major immunoreactants from IAA.

Table A3.2. IAA mAb immunoreactants and their HPLC retention times.

Cross-Reactant	Percent Cross-Reactivity, %	HPLC Retention Time, min.	Notes
Indole-3-acetic acid (unmethylated)	0.00	8.5	a
Indole-3-acetic acid	100.00	**19.6**	
Indole-3-acetamide	1.00	5.7	a
Indole-3-acetylglycine	57.90	8.3	
Indole-3-acetylalanine	1.50	10.6	a
Indole-3-acetone	5.20	13.9	
Indole-3-acetonitrile	1.00	16.2	a, b
Indole-3-acrylic acid	5.50	32.2	a
Indole-3-butryic acid	1.30	49.0	a

a: Found in nature.
b: Precursor or catabolite of IAA.

GC/MS-SIM

Low-End Sensitivity and Range of Linearity

Using the same GC/MS protocols used with ABA, low-end sensitivity for methyl-IAA

was estimated to be 50 pg/μL. This was based on the fact that the peak height for methyl-IAA was 3-10 times greater than the background noise. The range of linearity was apparent over a concentration range of 50 to 1000 pg/μL, with a correlation coefficient of 0.99 from the linear regression.

APPENDIX 4

SEED ORCHARD LOCATIONS

1. **Weyerhaeuser** (genotypes WA and WB)

   Franklin Brantley
   Production Manager
   Lyons Seed Orchard and Tree Improvement Facility
   Route 1, Box 119-A
   Lyons, GA 30436

2. **Union Camp** (genotypes 10-68 and 10-84)

   George Lowerts
   P.O. Box 216
   Rincon, GA 31326

3. **Westvaco** (genotypes 240 and 8)

   David Canavera
   Genetics and Biotechnology Center Leader
   Forest Research
   Box 1950
   Summerville, SC 29484